Functional analysis of a grapevine carotenoid cleavage dioxygenase (VvCCD1)

Justin Graham Lashbrooke

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Supervisor: Dr Philip R. Young

Co-supervisor: Prof. Melané A. Vivier

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Declaration

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Summary

The *Vitis vinifera* L. carotenoid cleavage dioxygenase 1 gene (*VvCCD1*) is a member of a structurally conserved gene family encoding enzymes that cleave multiple carotenoid substrates to form apocarotenoids. Carotenoid pigments are synthesised in the chloroplast where they are primarily involved in light harvesting and photo-protection during photosynthesis while apocarotenoids fulfill diverse roles that range from pollinator attractants to phytohormones. CCD1 cleaves carotenoids at specific double bond sites producing volatile apocarotenoids. These CCD1-derived apocarotenoids typically possess a fruity and floral aroma, thus making them desirable targets for metabolic engineering. CCD1 orthologues are highly homologous and have been isolated and characterised from a number of plant species, including *Arabidopsis*, tomato, rose, petunia, and grapevine.

VvCCD1 is localised to the cytosol and has been shown *in vitro* to cleave zeaxanthin and lutein resulting in 3-hydroxy-β-ionone. Expression of *VvCCD1* increases during berry ripening, peaking at véraison. Due to the impact that *VvCCD1* potentially has on the flavour and aroma of grape berries and therefore wine, this study aimed to characterise the specific enzyme action as well as the biological role that this enzyme plays in grapevine.

Expression of *VvCCD1* in carotenoid-accumulating *Escherichia coli* strains demonstrated cleavage of β-carotene at the 9,10 (9',10') position forming β-ionone; and lycopene at the 5,6 (5',6') and 9,10 (9',10') position, forming 6-methyl-5-hepten-2-one and pseudoionone, respectively. A transgenic grapevine population with modified *VvCCD1* expression was generated and genetically and metabolically characterised. The transgenic population consisted of lines in which *VvCCD1* was either overexpressed or silenced. Expression analysis of stable transformants showed a 12-fold range of *VvCCD1* expression relative to the wild-type.

HPLC analysis of the photosynthetic pigment content of the transgenic population necessitated the development and optimisation of a method for the extraction of pigments, specifically from grapevine. A number of parameters were identified and optimised, resulting in a method that provides accurate quantification of photosynthetic pigments from grape berries and leaves. Absolute quantification of the following major photosynthetic pigments

present in grapevine is now possible: chlorophyll *a*, chlorophyll *b*, lutein, β-carotene, zeaxanthin, antheraxanthin, violaxanthin and neoxanthin.

Data suggest that various levels of molecular control regulate carotenoid cleavage and apocarotenoid biosynthesis. The majority of lines stably transformed with a *VvCCD1* overexpression cassette exhibit post-transcriptional gene silencing. Expression analysis in these lines demonstrated that, despite the additional contribution of transgene-derived *VvCCD1* transcripts, the total *VvCCD1* transcript levels were not significantly higher than in wild-type lines. In lines where transgenic manipulation of *VvCCD1* expression was successful, subsequent analysis of carotenoids and apocarotenoids in leaf tissue showed no correlation between the measured metabolites and gene expression. The *in planta* action of VvCCD1 is presumably distinct from the observed *in vitro* activity due to the strict compartmentalisation required in photosynthetic leaf tissue preventing access of cytosolic VvCCD1 to the chloroplastic carotenoids.

Future studies on reproductive organs (grape berries) from the transgenic lines generated in this study will be of great importance in further elucidation of the *in planta* function of *VvCCD1*.

Opsomming

Die *Vitis vinifera* L. "carotenoid cleavage dioxygenase" 1 geen (*VvCCD1*) behoort aan 'n geenfamilie wat struktureel gekonserveerd is en kodeer vir ensieme wat verskeie karotenoïed substrate afbreek om apokarotenoïede te vorm. Karotenoïed pigmente word in die chloroplaste gesintetiseer waar hulle primêr betrokke is by lig-insameling, sowel as beskerming tydens fotosintese, terwyl apokarotenoïede diverse funksies in die plant verrig wat strek van aantrekking van stuifmeelverspreiders tot phytohormone. CCD1 breek karotenoïede by spesifieke dubbelbindingsetels af om vlugtige apokarotenoïede te vorm. Die apokarotenoïede wat van CCD1 afkomstig is besit tipies vrugtige en blomagtige aromas wat hul gesogte teikens maak vir metaboliese manipulering. CCD1 ortoloë is hoogs homoloog en is al geïsoleer en gekarakteriseer vanuit 'n verskeidenheid plantspesies wat *Arabidopsis*, tamatie, roos, petunia en wingerd insluit.

VvCCD1 is in die sitosol gelokaliseer en dit is vantevore gewys dat dit beide zeaxanthin en lutein *in vitro* kan afbreek om 3-hidroksi-b-ionoon te vorm. Die uitdrukking van *VvCCD1* vermeerder tydens korrel rypwording en bereik 'n maksimum tydens véraison. Weens die potensieële invloed van*VvCCD1* op die geur en aroma van druiwe, en dus wyn, is hierdie studie gerig op die karakterisering van die spesifieke ensiematiese aksie, sowel as die biologiese rol van hierdie ensiem in wingerd.

Uitdrukking van *VvCCD1* in *Escherichia coli* rasse wat karotenoïede versamel het getoon dat β-karoteen by die 9,10 (9',10') posisie afgebreek word om β-ionoon te vorm, en likopeen by die 5,6 (5',6') en 9,10 (9',10') posisie om onderskeidelik 6-metiel-5-hepteen-2-oon en pseudo-ionoon te vorm. 'n Transgeniese wingerd populasie is gegenereer met gewysigde *VvCCD1* uitdrukking en is geneties en metabolies gekarakteriseer. Die transgeniese populasie het bestaan uit lyne waar *VvCCD1* óf ooruitgedruk óf afgereguleer is. Uitdrukkings analise van die stabiele transformante het 'n 12-voudige reeks van *VvCCD1* uitdrukking getoon, relatief tot die wilde tipe.

HPLC analise van die fotosintetiese-pigment inhoud van die transgeniese populasie het die ontwikkeling en optimisering van 'n wingerd-spesifieke metode vir die ekstraksie van pigmente genoodsaak. 'n Aantal parameters is geïdentifiseer en geoptimiseer, en het gelei tot 'n metode wat akkurate kwantifisering van fotosintetiese pigmente in druiwe en wingerdblare

kan lewer. Absolute kwantifisering van die volgende belangrike fotosintetiese pigmente aanwesig in wingerd is nou moontlik: chlorophyll *a*, chlorophyll *b*, lutein, β-karoteen, zeaxantien, anteraxantien, violaxantien en neoxantien.

Data dui aan dat verskeie vlakke van molekulêre beheer die afbreking van karoteen en die biosintese van apokarotenoïede reguleer. Die meerderheid van die lyne wat stabiel getransformeer is met ‘n *VvCCD1* ooruitdrukkingskasset het na-transkripsioneleafregulering van die geen getoon. Uitdrukking analise van die lyne het gewys dat ten spyte van die addisionele transgeniese *VvCCD1* transkripte, die totale *VvCCD1* transkripvlakke nie beduidend hoër was as dié van die wilde-tipe lyne nie. In die lyne waar transgeniese manipulasie van *VvCCD1* uitdrukking wel suksesvol was, het verdere analise van die karotenoïed en apokarotenoïed vlakke in blaarweefsel geen korrelasie getoon tussen die metaboliete en *VvCCD1* uitdrukking nie. Die *in planta* aktiwiteit van VvCCD1 is vermoedelik anders as die *in vitro* aktiwiteit weens die streng kompartementalisering benodig in fotosintetiese blaarweefsel, wat verhoed dat die sitosoliese VvCCD1 toegang het tot die chloroplastiese karotenoïede.

Toekomstige bestudering van die reproduktiewe organe (druiwe) van die transgeniese lyne wat in hierdie studie gegenereer is sal belangrik wees in die verdere verduideliking van die *in planta* funksie van VvCCD1.

Biographical sketch

Justin Lashbrooke was born in 1983 in Cape Town, South Africa and attended Wynberg Boys' High School where he matriculated in 2001. He completed a BSc in Microbial Biotechnology followed by a BSc(Hons) in Wine Biotechnology at Stellenbosch University in 2005 and 2006, respectively.

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Preface

This thesis is presented as a compilation of five chapters. Each chapter is introduced separately and is written according to the style of the journal Australian Journal of Grape and Wine Research to which Chapter 3 has been submitted for publication.

Chapter 1 **General introduction and project aims**

Chapter 2 **Literature review**
The role of plant carotenoid cleavage dioxygenases in apocarotenoid production

Chapter 3 **Research Results**
The development of a method for the extraction of carotenoids and chlorophylls from grapevine leaves and berries for HPLC profiling

Chapter 4 **Research Results**
Functional characterisation of a *Vitis vinifera* carotenoid cleavage dioxygenase, *VvCCD1*

Chapter 5 **General discussion and conclusions**

I hereby declare that I was the primary contributor with respect to the experimental data presented in the multi-author manuscripts presented in the research chapters (Chapters 3 and 4). My supervisors, Prof. M. A. Vivier and Dr P. R. Young, were involved in the conceptual design of the study and critical evaluation of the manuscript (Chapter 3 and 4). Mr A. E. Strever acquired field data relevant to the leaf characterisation (viticultural measurements) discussed in Chapter 3. Ms C. Stander acquired field data relevant to the berry characterisation (sugar and organic acid measurements) discussed in Chapter 3. Where relevant, technical assistance is acknowledged in the specific research chapters.

Contents

Chapter 1

General introduction and

project aims

1.1 Introduction

Plant carotenoid cleavage dioxygenase (CCD) enzymes are a family of enzymes that catalyse the oxidative cleavage of numerous carotenoids. Cleavage results in the formation of apocarotenoids, compounds which perform a number of important biological roles in plants. Plant carotenoid biosynthesis occurs in the plastid where carotenoids are primarily involved in photosynthesis. Here they are bound in complexes with chlorophylls in the plastidial membranes. They assist during harvesting of light energy and as a protective mechanism for chlorophylls by dissipating excess energy and preventing photo-oxidative damage through the scavenging of free radicals (Demmig-Adams & Adams 1996).

The carotenoid-derived apocarotenoids fulfil a number of roles in plants such as hormones, pollinator attractants and flavour and aroma compounds. Several are of economic importance and are commonly extracted and used as flavourants and colourants in the food and cosmetic industry, while others create the distinct flavour and aroma of agriculturally important flowers and fruits. Some important apocarotenoids that result from the enzymatic cleavage of carotenoids by the CCD enzyme family include the phytohormone abscisic acid (ABA) (Liotenberg et al. 1999), the volatile floral aroma compound β-ionone (Vogel et al. 2008) and the hormone-like strigolactone, which inhibits shoot branching (Dun et al. 2009).

Analysis of plant carotenoids has proved challenging as these compounds are highly susceptible to degradation by light, oxygen and heat (Oliver & Palou 2000). This, along with the diversity seen between plant tissues, has resulted in a number of differing techniques existing for carotenoid quantification. Previously a profiling method for the quantification of pigments in *Arabidopsis thaliana* was optimised (Taylor et al. 2006), but its implementation to grapevine tissue was not successful.

The CCD gene family in plants consists of nine members, all encoding genes that catalyse the cleavage of carotenoids. Members of the family include the 9-*cis*-epoxydioxygenases (*NCEDs*), encoding enzymes that cleave 9-*cis*-epoxycarotenoid substrates leading to the formation of ABA (Liotenberg et al. 1999, Schwartz et al. 2003); *CCD1s* which code for enzymes that cleave a broad range of carotenoids forming volatile aroma compounds (Vogel et al. 2008); *CCD4s* which encode enzymes catalysing the cleavage of carotenoids forming the aroma and pigment compounds of the spice, saffron and the colourant, bixin (Huang et al.

2009b); and *CCD7s* and *CCD8s* which encode enzymes that catalyse the sequential cleavage of carotenoids forming strigolactone, a hormone-like compound that inhibits shoot branching in plants (Booker et al. 2004, Dun et al. 2009). The broad range of apocarotenoids produced by CCD cleavage and the subsequently diverse biological roles performed by these compounds demonstrates the importance of CCDs to plants in functions as diverse as drought tolerance, attractors of pollinators, and growth and developmental regulation.

CCD1 orthologues encode enzymes able to catalyse the symmetrical cleavage of numerous carotenoids, resulting in the formation of volatile apocarotenoids including geranylacetone, pseudoionone, β-ionone, 3-hydroxy-β-ionone, α-ionone and 6-methyl-5-hepten-2-one (Holger et al. 2006, Mathieu et al. 2005, Vogel, et al. 2008). These CCD1-derived apocarotenoids possess fruity and floral aromas (Mendes-Pinto 2009). While *CCD1* is expressed in leaves, higher expression levels are seen in flowers and fruits with a marked increase typically occurring during fruit ripening (Huang et al. 2009a, Simkin et al. 2004b). CCD1-derived apocarotenoids therefore contribute to the flavour and aroma of a number of cultivated crops, ensuring that the metabolic engineering of apocarotenoids is of interest to the agricultural industry.

Substrate specificity of CCD1 has largely been determined either through *in vitro* enzymatic assays or through the expression of *CCD1* genes in carotenoid-accumulating strains of *Escherichia coli*. The high degree of homology between *CCD1* orthologues suggests that they should exhibit similar substrate specificities (Simkin et al. 2004a, Vogel et al. 2008). Grapevine (*Vitis vinifera* L.) CCD1 has only been demonstrated to cleave zeaxanthin and lutein (forming the volatile 3-hydroxy-β-ionone) in *in vitro* enzymatic assays (Mathieu et al. 2005). Further characterisation is required to determine the substrate range of *VvCCD1*.

Although a large amount of work has been done investigating CCD1 enzymes, the *in planta* function(s) of *VvCCD1* (like its orthologues) is still poorly described. Mathieu et al. (2005) found that expression of *VvCCD1* in ripening berries preceded the formation of volatile apocarotenoids by one week. The cause of this disparity between *VvCCD1* transcript levels and known enzyme action remains unknown and requires further investigation to determine the level(s) of molecular control.

CCD1 expression levels have been manipulated, through transgenic RNAi techniques, in *Lycopersicon esculentum* (tomato) fruit (Simkin et al. 2004a) and in *Medicago truncatula* (barrel clover) roots (Floß et al. 2008). The findings from these *in planta* studies were, however, inconsistent with the previously published *in vitro* activity data of the respective enzymes (Floß et al. 2008). Carotenoid concentrations were unaffected by a decrease in *CCD1* transcript levels, while apocarotenoid concentrations were only weakly correlated to *CCD1* expression. These results further highlight the complexity of the molecular control exerted on this enzyme.

1.2 Project aims

The aim of this study is the *in vitro* and *in planta* functional characterisation of *VvCCD1*. This includes determination of the substrate range for VvCCD1, which is likely to be broader than currently described; and the generation, and subsequent genetic and metabolite analyses to characterise transgenic grapevine with altered levels of *VvCCD1* expression. Due to the long youth phase and growth cycle of grapevine, this study will only focus on the analysis of vegetative tissue. Techniques and technologies will, however, be optimised for both grapevine leaves and berries, since the analysis will be extended to reproductive tissue. Biochemical analysis of vegetative tissue (leaf) will include HPLC separation and quantification of photosynthetic pigments and GC/MS analysis of volatile apocarotenoids. This data should contribute to our fundamental understanding of the biological role that *VvCCD1* plays in grapevine. Of particular interest is the contribution that this gene makes to the flavour and aroma profile of grapevine. According to 2007 statistics of the Food and Agriculture Organization (FAO), grapevine is the most widely cultivated fruit crop in the world (http://www.fao.org). These grape-derived flavour and aroma apocarotenoid compounds contribute to the ultimate quality of table grapes and wine (Mendes-Pinto 2009). The characterisation of VvCCD1 is therefore required to understand the mechanisms contributing to the final flavour and aroma of grapes and wine. A fundamental understanding of the molecular mechanisms underlying the formation of these compounds in grapevine will facilitate the improvement of the flavour and aroma of wine. To this end the following specific aims were formulated for this study:

i) The *in vitro* characterisation of the substrate specificity of VvCCD1 through the transformation of carotenoid-accumulating *E. coli* strains with *VvCCD1* expression cassettes and subsequent measurement of the apocarotenoids formed;
ii) *Agrobacterium*-mediated transformation of *V. vinifera* cv. Sultana with *VvCCD1* overexpression cassettes and *VvCCD1* silencing cassettes, and the establishment of a population of genetically characterised transgenic lines;
iii) Development and optimisation of a photosynthetic pigment extraction and HPLC separation and quantification protocol for grapevine tissues and the subsequent characterisation of the pigment profiles in the leaves of the transgenic grapevine lines in comparison with wild-type lines;
iv) Analysis via GC/MS of volatile apocarotenoid formation in the leaves of the transgenic lines in comparison with wild-type lines; and
v) Evaluation of the correlation between expression of *VvCCD1* and its known enzymatic substrates (carotenoids) and products (apocarotenoids) in the leaves of the transgenic population.

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Chapter 2

Literature review

The role of plant carotenoid cleavage dioxygenases in apocarotenoid production

2.1 Introduction

2.1.1 Carotenoids

Carotenoids are a group of over 700 naturally occurring red, orange and yellow lipophilic isoprenoid pigments (Britton 1993, Cunningham 2002). Synthesis of carotenoids occurs predominantly in photosynthetic organisms including plants, algae and cyanobacteria. The C_{40} carotenoids are synthesised through the condensation of two C_{20} geranylgeranyl diphosphate (GGPP) molecules (Britton 1995). The primary function of plant carotenoids is to serve as accessory pigments during photosynthesis, resulting in the relative carotenoid concentration being highly conserved in photosynthetic tissue. Lutein constitutes 45% of the total, β-carotene 25-30%, violaxanthin 10-15%, and neoxanthin 10-15% (Liotenberg et al. 1999). During photosynthesis carotenoids participate in light harvesting by absorbing light in the range of 450-570 nm and transferring the energy to chlorophyll. Carotenoids are also able to protect the photosynthetic apparatus from damage by enabling the dissipation of the excess absorbed energy as heat and preventing photo-oxidation through the scavenging of free radicals (Demmig-Adams & Adams 1996).

In flowers and fruits the carotenoid composition and the specific concentrations of carotenoid are unique to individual plant species (Tanaka et al. 2008). In non-photosynthetic organs such as flowers and fruits, where chlorophyll concentrations are lower, the coloured carotenoids are more visible. The chromoplasts of flowers and fruits accumulate high concentrations of carotenoids which serve to attract pollinators and facilitate seed dispersal, respectively (Demmig-Adams & Adams 1996).

2.1.2 Apocarotenoids

Carotenoids are susceptible to oxidative cleavage resulting in the formation of apocarotenoids. This group of terpenoid compounds is structurally diverse and fulfils a wide variety of functions throughout nature with many having an important industrial value (reviewed in Giuliano et al. (2003)). One of the most important apocarotenoids is the phytohormone, abscisic acid (ABA) involved in developmental regulation and the response to drought stress. Another hormone-like apocarotenoid performing a signalling role in plants is strigolactone, which inhibits shoot branching (Dun et al. 2009). Furthermore, apocarotenoids contribute to the flavour, aroma and pigment profile of fruits and flowers, which in turn act as attractants of pollinators. Economically important apocarotenoids include the flavourant β-ionone, crocin which significantly contributes to the red colour of saffron, and bixin which is

used as a colourant in foods and cosmetics. Additionally, these compounds regularly form the basis for the flavour and aroma of agronomically important crops.

2.1.3 CCDs: discovery, classification, engineering prospects

Unspecific oxidative cleavage of carotenoids can occur in a number of chemical, thermal or enzymatic processes to produce apocarotenoids. Carotenoid cleavage dioxygenases (CCDs) are a group of plant enzymes that are able to catalyse cleavage of a variety of carotenoids at specific double bond sites, permitting the plant to tailor its apocarotenoid content (Figure 1) (Auldridge et al. 2006).

The first characterised member of the plant CCD family was *VIVIPAROUS14* (*VP14*) found in maize (Schwartz et al. 1997). Analysis of viviparous maize seeds deficient in ABA led to the cloning of *VP14*. Enzymatic assays performed with recombinant VP14 displayed its ability to cleave 9-*cis*-epoxycarotenoids which is the first committed step to ABA synthesis in plants (Schwartz et al. 2003). Sequence comparisons with *VP14* led to the initial discovery of the *CCD* gene family in *Arabidopsis thaliana* followed by other higher plants. This gene family consists of nine members, all of which encode enzymes that are able to catalyse oxidative cleavage of carotenoids. Five members of the *CCD* gene family are involved in the synthesis of ABA via the cleavage of 9-*cis*-epoxycarotenoids (Tan et al. 2003). This *CCD* gene sub-family are named 9-*cis*-epoxycarotenoid dioxygenases (*NCED*s) and consist of *NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9*. The remaining four genes are given the generic name *CCD*, so as to distinguish them from the 9-*cis*-epoxycarotenoid cleaving enzymes. Of these four, *CCD7* and *CCD8* are involved in the production of the signalling molecule, strigolactone, while *CCD1* and *CCD4* code for enzymes with a broad range of substrate specificity, producing a number of volatile norisoprenoids (Booker et al. 2004, Ohmiya et al. 2006, Vogel et al. 2008). Along with the growing understanding of the metabolic engineering of carotenoid biosynthesis (reviewed in (Giuliano et al. 2008), the discovery and characterisation of CCDs that catalyse specific reactions to form apocarotenoid products has resulted in the possibility of metabolically engineering these apocarotenoids. It should be noted, however, that difficulties observed in carotenoid engineering are likely to be encountered when attempting to engineer apocarotenoids. These include limited knowledge of the signalling mechanisms controlling plant carotenoid biosynthesis, and the crosstalk between the carotenoid biosynthetic pathway and other metabolic pathways (Giuliano et al. 2008).

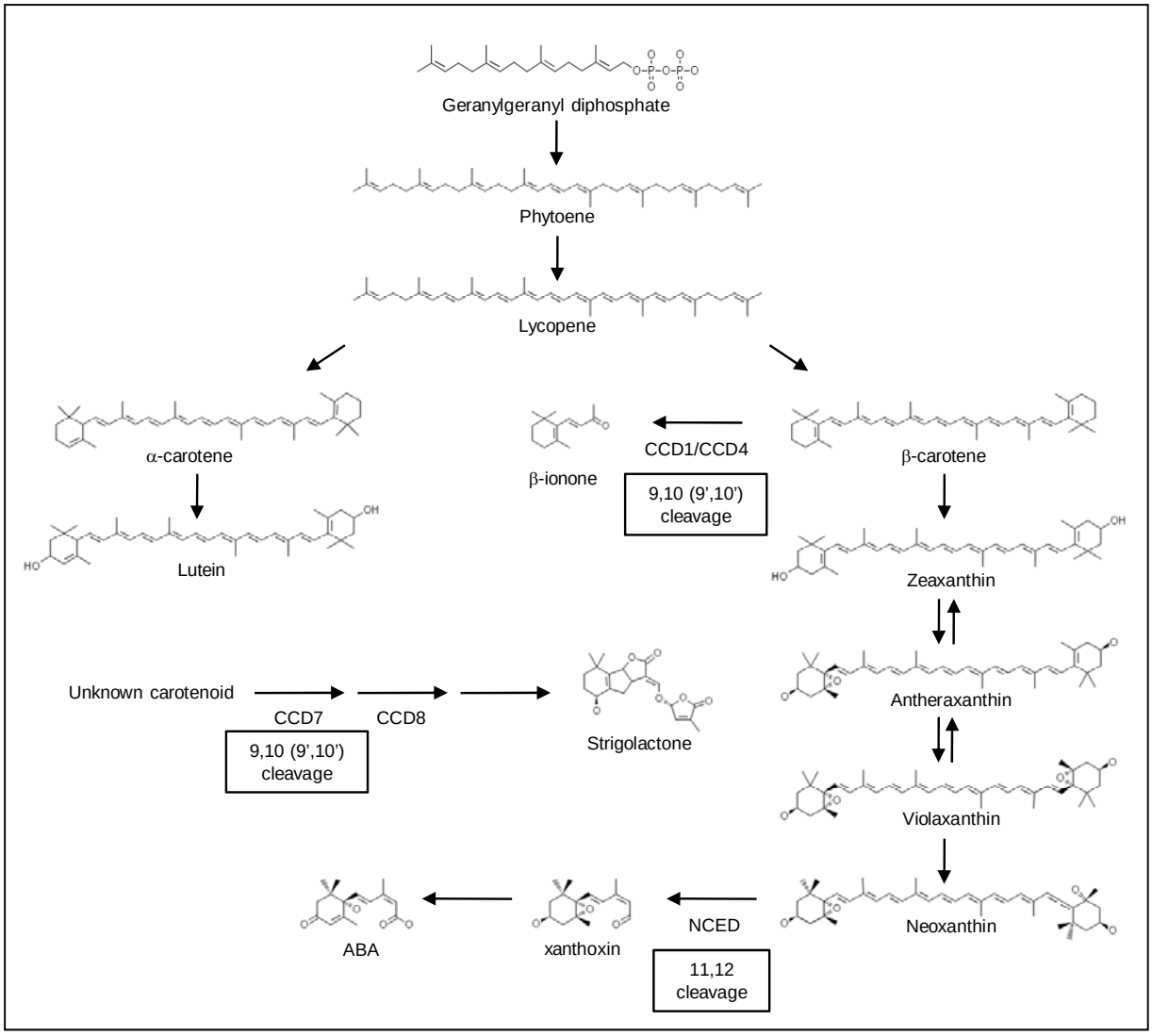

Figure 1. Carotenoid pathway and apocarotenoids formation. A schematic showing a simplified carotenoid pathway found in higher plants and a number of the potential apocarotenoids produced from cleavage by carotenoid cleavage dioxygenases.

2.2 CCD biochemistry

2.2.1 Mode of action of CCDs

While CCDs catalyse the cleavage of carotenoids at specific double bonds they are not so discerning when it comes to substrate specificity. An example of this can be seen in the preference of CCD1 to cleave at the 9,10 (9',10') double bond of numerous carotenoids including lycopene, β-carotene, δ-carotene, zeaxanthin and lutein (Vogel et al. 2008); while NCED cleaves neoxanthin and violaxanthin at the 11,12 double bond (Tan et al. 2003). Cleavage can be asymmetrical, as in the case of NCEDs and CCD7, or symmetrical as in the case of CCD1. The symmetrical cleavage shown by CCD1 orthologues has resulted in the speculation that this enzyme acts as a dimer (Schwartz et al. 2001).

All members of the CCD family are able to cleave a C-C double bond with the addition of molecular oxygen (Kloer & Schulz 2006). Cleavage results in the formation of an aldehyde and a ketone. Although apocarotenoid formation occurs via nonspecific oxidation it is likely that the biologically active apocarotenoids in plants are generated via site-specific CCD-mediated cleavage.

Despite the *in vitro* activity of CCDs requiring excess Fe^{2+} it has not been demonstrated that iron participates stoichiometrically in the reaction, but rather as a co-factor (Kloer & Schulz 2006). The electrons present in the products formed can be accounted for by the carotenoid and the oxygen molecule.

2.2.2 Subcellular localisation of CCD

The carotenoid substrates of CCD enzymes are most often large, lipophilic molecules. They are therefore not commonly found in the cytosol of the plant cell, but rather embedded in the plastid membranes together with chlorophylls (Cunningham 2002). In plants the carotenoid biosynthetic pathway exists exclusively in the plastids. Here the various carotenoids are synthesised and are typically bound to the thylakoid membranes together with chlorophyll (REF). Both chloroplast and chromoplast membranes house carotenoids. The majority of CCD enzymes are therefore targeted to these plastids (Auldridge et al. 2004). This has been confirmed with *in situ* western blots as well as the prediction of plastidial signal peptides present in all members of the plant CCD family, except CCD1 which is targeted to the cytosol (Vogel et al. 2008). The transport of CCD proteins across the plastid membranes has been observed in some cases to modify the proteins, resulting in two versions of these enzymes (Endo et al. 2008). This compartmentalisation of CCD substrates from the cytosol creates an interesting problem for the metabolic engineering of apocarotenoids in plants.

2.2.3 The structure of CCD enzymes

The structure of plant CCDs has not been viewed directly, but the structure of a family member, apocarotenoid-15,15'-oxygenase (ACO), from *Synechocystis* sp. PCC 6803 has been resolved by (Kloer et al. 2005) (Figure 2). A high degree of similarity between the amino acid sequences of ACO and plant CCDs at important structural regions of the proteins, allows for relevant structural predictions of CCDs to be made (Kloer & Schulz 2006). The tertiary structure of ACO forms a seven-bladed “propeller”, with each propeller being formed from antiparallel β-sheets. Four histidine residues occur at the propeller axis. These residues are

conserved throughout the CCD enzyme family (Kloer & Schulz 2006). An Fe^{2+} cation is held in place by the histidine residues and forms the active centre of the enzyme (Kloer & Schulz 2006). Loops joining the propeller blades form a dome over this active centre. A tunnel through ACO passes the active centre of the protein. The propeller provides a rigid structure for the loops from which the tunnel is formed. A second tunnel enters ACO perpendicular to the first but stops just short of the active centre, allowing for the reactant, dioxygen, to reach the active centre. This tunnel is formed by the propeller structure so is likely to be conserved throughout the CCD family. A non-polar patch consisting of projecting leucine residues on the surface of ACO facilitates protein attachment to non-polar membranes (Kloer & Schulz 2006).

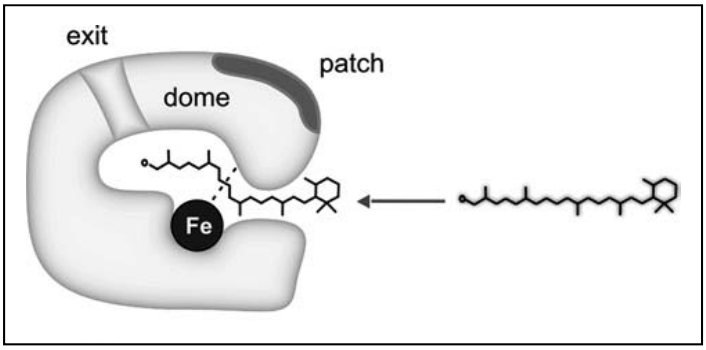

Figure 2. Schematic sketch of apocarotenoid cleavage by ACO. This figure, as shown in Kloer & Schulz (2006), depicts the tunnel entrance, which determines the substrates that the enzyme accepts, the active site, which contains an Fe2+ ion essential for cleavage, and the non-polar patch, allowing binding to non-polar membranes.

Sequence comparisons between members of the CCD gene family performed by (Kloer & Schulz 2006) show that the residues that form the propeller structure are the most highly conserved, while the residues coding for the loops are divergent. Substrate specificity is determined by the tunnel structure, and therefore the peptide strands that make up the loops. Modification in these sequences influences the length, width and entrance size of the tunnel. This alteration of tunnel morphology enables control over which substrates can enter the tunnel and at what position they are cleaved without affecting the rigid propeller structure of the enzyme. The inner surface of the tunnel consists of many non-polar residues accommodating the hydrophobic substrates. Through evolution these loops have diverged resulting in the wide range of substrate specificity seen throughout the CCD family. However, the low level of conservation between the peptide sequences that form the loops

impedes modelling of the tunnel in ACO-related enzymes, such as plant CCDs. The structure of more members of the CCD family will have to be determined in order to explain the apparent substrate promiscuity of these enzymes (Kloer & Schulz 2006).

2.3 Biological roles of CCDs

2.3.1 The NCEDs and their role in ABA biosynthesis

Five NCEDs have been identified in *Arabidopsis thaliana* (Tan et al. 2003). They take their name from their nine-*cis*-epoxycarotenoid substrates (e.g. neoxanthin and violaxanthin). Cleavage of these substrates by NCED at the 11,12 double bond is the first committed step in abscisic acid (ABA) biosynthesis, and has shown to be the key regulatory step in this process (Thompson et al. 2007). NCED-mediated cleavage results in the formation of xanthoxin, which is further oxidised by xanthoxin dehydrogenase (ABA2) and *Arabidopsis* aldehyde oxidase 3 (AAO3) forming ABA (Figure 3) (Melhorn et al. 2008).

ABA performs a crucial role during the seed development of many plants and in the plant's response to abiotic and biotic stresses, including drought, salt, temperature and pathogen attack (Barrero et al. 2006, Endo et al. 2008, Iuchi et al. 2001). One of the most studied roles of ABA is its role in the regulation of the plant's response to drought stress. The biosynthesis of ABA in some plants leads to a systemic response by the plant triggering adaptation to drought conditions (Endo et al. 2008). Osmotic stress in the plant's roots will result in an increase in ABA concentration above ground. The consequent accumulation of ABA in plant cells initiates the transcription of a number of drought-inducible genes. A well studied example is the closure of the stomata in order to reduce transpiration (Melhorn et al. 2008, Soar et al. 2004).

In *A. thaliana* all five *NCED*s (*NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9*) have been shown to be involved in ABA biosynthesis. *AtNCED3*, however, has been shown to be primarily responsible for the biosynthesis of ABA in response to dehydration stress (Ren et al. 2007). The remaining *NCED*s are expressed in response to developmental changes. In *Arabidopsis*; under drought conditions, AtNCED3 has been shown to be exclusively localised to vascular parenchyma cells (Endo et al. 2008). The presence of AtNCED3 protein in these cells is only observed in water-stressed plants in conjunction with the downstream functioning enzymes (AtABA2 and AAO3), resulting in the formation of ABA. In turgid plants, however, while AtABA2 and AtAAO3 are still present in the vascular tissue,

AtNCED3 is not. During drought conditions in *Arabidopsis* AtNCED3 is therefore a key regulatory step for ABA biosynthesis.

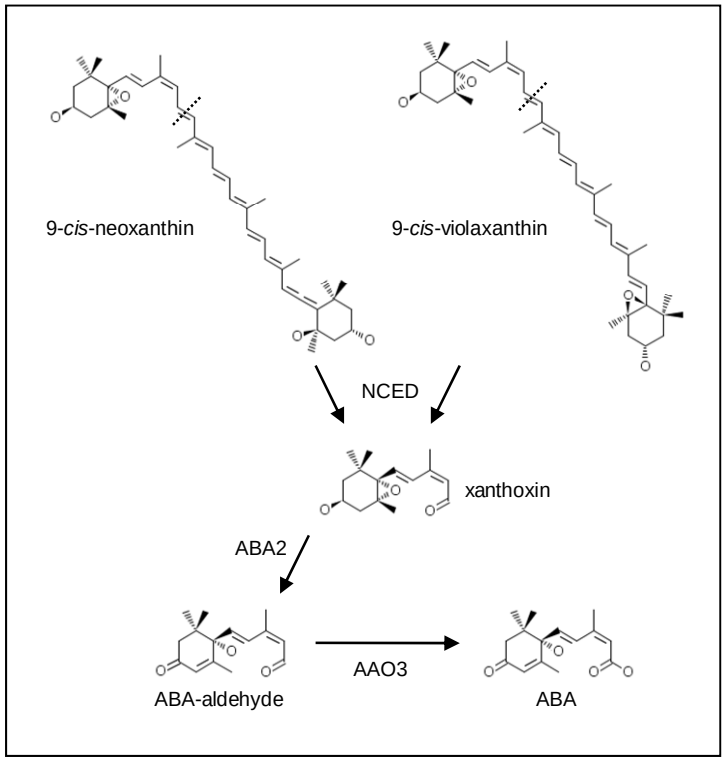

Figure 3. Shematic showing NCED-mediated formation of ABA. Cleavage of 9-*cis*-epoxycarotenoids at the 11,12 double bond by nine-*cis*-epoxycarotenoid dioxygenase (NCED) leads to the formation of ABA via xanthoxin, mediated by xanthoxin dehydrogenase (ABA2) and *Arabidopsis* aldehyde oxidase 3 (AAO3).

NCED orthologues discovered in other higher plants have been numbered chronologically. This, in conjunction with the fact that many higher plants appear to possess only one or two *NCEDs* as opposed to the five in *Arabidopsis*, has resulted in the naming of the *NCED* orthologues being inconsistent between plant species.

Plant organs developmentally regulated by ABA biosynthesis exhibit synchronised expression of *NCED*. This has been observed in reproductive structures and lateral root initials (Nitsch et al. 2009, Zhang et al. 2009). In tomato ovaries *LeNCED1* has been shown to strongly regulate the level of ABA, in a process involving phytohormone crosstalk (Nitsch et al. 2009). An increase in ABA is observed in maturing tomato ovaries, with mature ovaries showing a relatively high level of ABA. Upon pollination a decrease in ABA is observed.

This decrease in ABA occurs due to a decrease in *NCED* expression, and an increase in an ABA hydroxylase gene (*SlCYP707A1*) which codes for an enzyme that catalyses an ABA catabolic reaction. After pollination an increase in auxin concentrations in the ovary causes an increase in gibberellic acid, both these hormones inhibit *NCED* expression while auxin stimulates the expression of *SlCYP707A1* resulting in ABA catabolism.

Another example of the involvement of *NCED* in hormonal crosstalk and the developmental regulation exerted by ABA has been observed in peach fruits and grape berries (Zhang et al. 2009). It was shown that expression of *PpNCED1* (peach) and *VvNCED1* (grapevine) at the beginning of fruit ripening resulted in ABA biosynthesis. An increase in ethylene followed the increase in ABA. Use of carotenoid pathway inhibitors to block the production of *NCED*-derived ABA caused a suppression of ethylene followed by delayed fruit ripening in both peach and grapevine. This observation led Zhang et al. (2009) to conclude that NCED is also able to initiate ripening in these fruits by acting as a stimulant for ethylene production via the production of ABA.

In a number of plants ABA is responsible for the regulation of development and dormancy of seeds (Liotenberg et al. 1999). NCED-mediated carotenoid cleavage has been observed to be the primary mechanism for ABA synthesis in developing *Arabidopsis* seeds. *NCED5* and *NCED6* are expressed in the endosperm and embryonic tissues while *NCED3* is expressed primarily in the maternal tissues of seeds (Karssen et al. 1983).

Transcriptional regulation of ABA biosynthesis has been observed during developmental regulation, as well as during stress responses. In addition, regulation can also occur post-translationally (Endo et al. 2008). In *Arabidopsis* the various NCEDs all show distinct variation in their ability to bind to thylakoid membranes. While all NCEDs are localised to the plastid, a physical separation between stroma-bound and carotenoid-containing membrane-bound forms exists. (Endo et al. 2008) identified two forms of AtNCED3 via western blot analysis, a 64 kD and a 56 kD form. The 64 kD form was observed to be bound to the thylakoid, while the 56 kD form was bound to the stroma. Only the levels of the thylakoid-bound form showed a strong positive correlation with dehydration stress and increased ABA levels. The stroma-bound NCED could still be detected after depletion of ABA. An amphipathic domain at the amino terminus of the NCED proteins facilitates

thylakoid membrane binding, and it is thought that this domain is removed during import into the chloroplast resulting in a stroma-binding form of NCED (Endo et al. 2009).

2.3.2 CCD7 and CCD8 and their role in the regulation of shoot branching

Shoot branching in plants is known to be controlled by phytohormones including auxins and ethylene. Auxins produced by the apical meristem stimulate high levels of ethylene around lateral buds, inhibiting lateral shoot development and maintaining apical dominance (Ferguson & Beveridge 2009). These hormones allow the plant to react to a wide variety of environmental and developmental influences resulting in the hugely diverse phenotypes of genetically identical plants. Shoot branching occurs in the absence of auxins through outgrowth of previously dormant axillary buds.

CCD7 and *CCD8* play an important role in the regulation of lateral shoot growth and development. These genes, also referred to as *MAX3* and *MAX4* (more axillary branching), code for proteins localised to the plastid (Booker et al. 2004). Investigation into the role of *CCD7* and *CCD8* during lateral shoot development was performed by REF. Through grafting of *Arabidopsis* root and scion loss-of-function mutants they were able to show that CCD7 and CCD8 produce an apocarotenoid product subsequently identified as strigolactone (Booker et al. 2005). Strigolactone leads to inhibition of shoot branching (or the inhibition of axillary bud outgrowth) only when both gene products are expressed in the same location (root or scion) (Sorefan et al. 2003).

Work by (Turnbull et al. 2002) demonstrated that strigolactone can be transported acropetally (from base to apex) via the xylem. Inhibition of shoot branching was only observed in scion tissue expressing *MAX1* (encoding a cytochrome P450 monooxygenase), suggesting that strigolactone produced in the roots, requires further modification for its inhibitory action on shoot branching (Booker et al. 2005).

Expression studies of *CCD7* and *CCD8* show that the genes are expressed primarily in root tissue, while *MAX1* expression is localised to vascular tissue throughout the plant (Booker et al. 2005). These data indicates that strigolactone is produced mainly in the roots and that it is able to act as a long range signalling molecule. It has been shown that very small quantities are able to restore wild-type branching inhibition to plants not expressing *CCD7* and *CCD8* (Yoneyama et al. 2009).

This effect on the branching phenotype by *CCD7* and *CCD8* has been observed in a number of plants. In pea (*Pisum sativum* L.) and petunia (*Petunia hybrida*) a mutation in the *RMS1* gene and the *DAD1* gene, respectively leads to increased branching in both plants (Alder et al. 2008, Sorefan et al. 2003). *RMS1* and *DAD1* are both *CCD8* orthologues. Application of auxin (known to inhibit shoot branching) to these mutants does not restore the wild-type phenotype, suggesting that CCD8 acts downstream of an auxin-mediated response for inhibition of shoot branching (reviewed in (Ongaro & Leyser 2007)). In grafting experiments similar to those performed with *Arabidopsis* (Brewer et al. 2009), normal shoot branching is restored in both pea and petunia *ccd8* mutants when grafted to wild-type root tissue. This corroborates the finding that root-derived strigolactone is involved in inhibiting shoot branching and is possibly conserved throughout higher plants ((Sorefan et al. 2003, Dun et al. 2009, Foo et al. 2005).

In rice, possessing a mutated *CCD7* gene, an increase in tillers is observed as well as a dwarf phenotype (Arite et al. 2009). The increase in tillering in the monocotyl rice can be viewed as the equivalent to shoot branching in dicots, with strigolactone product inhibiting both processes. This enforces the hypothesis that strigolactone-mediated shoot inhibition is a highly conserved mechanism occurring in monocots and dicots.

Strigolactone also promotes hyphal branching of symbiotic arbuscular mycorrhizal fungi during root colonisation and the germination of parasitic weeds (Akiyama et al. 2005, Bouwmeester et al. 2007).

The cleavage of linear and cyclic carotenoids *in vitro* by CCD7 and CCD8 has been demonstrated by (Schwartz SH 2004). CCD7 cleavage occurs asymmetrically at the 9,10 double bond. In the case of β-carotene, CCD7 cleavage results in the formation of the C_{13}-ketone, β-ionone, and the C_{27}-aldehyde, 10'-apo-β-carotenal. CCD8 is then able to cleave 10'-apo-β-carotenal at its 13,14 double bond producing a C_{18}-ketone, 13-apo-β-carotene. Expression of *MAX1* is then thought to be necessary for metabolic bioconversion of 13-apo-β-carotene to strigolactone, as *max1* mutants did not show inhibition of shoot branching (Booker et al. 2005).

In rice an additional gene (encoding α/β hydrolase enzyme) named *DWARF14* (*D14*) has been shown to be necessary to elicit a strigolactone-dependant response (Arite et al. 2009).

The *d14* mutant possesses increased shoot branching usually observed in strigolactone-deficient strains. Application of exogenous strigolactone, however, does not restore the wild-type phenotype indicating that D14 functions downstream of strigolactone synthesis. It is unknown if D14 acts as a signalling component of the strigolactone pathway or is involved in the bioconversion of strigolactone to a bioactive hormone.

The importance of *CCD7* and *CCD8* is significant considering they encode enzymes able to catalyse the first committed steps in the biosynthesis of the hormone-like molecule, strigolactone. The long range signalling ability of the strigolactone pathway (or strigolactone specifically), as well as the possible role of strigolactone in hormone crosstalk with auxins and cytokinins as discussed by (Ferguson & Beveridge 2009) necessitates more study into the pathway.

2.3.3 CCD4 and its role in pigment and flavour formation

CCD4 orthologues have been characterised in a number of plant species, where they appear to be involved in the tailoring of carotenoid-derived pigments as well as the production of volatile norisoprenoids. These pigments and flavour compounds are often of economic importance, and include saffron and bixin, while in chrysanthemums CCD4 controls petal colour (Ohmiya et al. 2006).

As is the case with the majority of CCD enzymes, CCD4 is localised to the plastid. Although cleavage by CCD4 is not as well characterised as the rest of the CCD gene family, all the orthologues characterised thus far exhibit symmetrical cleavage of a variety of carotenoids at various double positions (Auldridge et al. 2006). In *Arabidopsis*, *AtCCD4* is expressed during petal differentiation and anthesis (Huang et al. 2009).

In *Crocus sativus* (saffron) CCD4-derived apocarotenoids contribute to the orange-yellow colour, flavour and aroma of saffron spice (Bouvier et al. 2003). These include crocin, crocetin, picrocrocin, and safranal. The orthologue of *CCD4* in *C. sativus* is zeaxanthin cleavage oxygenase (*CsZCD*), named for its ability to cleave zeaxanthin at the 7,8 (7'8') double bonds (Bouvier et al. 2003). CsZCD expressed in β-carotene accumulating *E. coli* strains results in the formation of β-ionone formation (Rubio et al. 2008). This indicates that the enzyme is also able to cleave at the 9,10 (9',10') double bonds.

The *CCD4* orthologue found in *Bixa orellana* (*BoLCD*) catalyses the cleavage of lycopene at the 5,6(5'6') double bonds (Bouvier et al. 2003) . *B. orellana* is a small tropical tree cultivated for its fruit, the seeds of which contain the red pigment, bixin. Bixin is used as both food colourant and flavouring agent. Lycopene cleavage by BoLCD followed by the action of bixin aldehyde dehydrogenase (BoBADH) leads to the formation of bixin as confirmed by the expression of *BoLCD* and *BoBADH* in lycopene-accumulating *E. coli* strains results in the production of bixin (Bouvier et al. 2003)

Chrysanthemum petals are commonly white in colour, but infrequently exhibit a yellow colour. White Chrysanthemums express all the genes necessary for carotenoid biosynthesis, however, carotenoid levels are controlled by CmCCD4 (Kishimoto & Ohmiya 2006). Loss-of-function mutations of *CmCCD4* give rise to yellow, carotenoid-rich petals. Transgenic reduction of *CmCCD4* mRNA levels in Chrysanthemums was performed by (Ohmiya et al. 2006). The RNAi strategy implemented led to reduced cleavage of carotenoids and their subsequent accumulation resulted in coloured petals. The ability to tailor carotenoid composition of petals and thus the ability to manipulate the outward phenotype of ornamental flowers has potential to contribute to a lucrative industry. An orthologue of CCD4 has also been found in roses (*Rosa damascena*) (Huang et al. 2009). Transcription of *RdCCD4* is seen almost exclusively in the flowers of roses (Huang et al. 2009). Fifteen different apocarotenoid volatiles have been identified in rose petals suggesting that this CCD4 homologue is able to cleave a broad range of carotenoids.

2.3.4 CCD1 and its role in the formation of flavour compounds

CCD1 orthologues from a variety of plant species cleave a number of carotenoids symmetrically at the 9,10 (9',10') double bond position. One exception to this rule has been observed in *Lycopersicon esculentum* (tomato) where LeCCD1 is also able to cleave lycopene at the 5,6 (5',6') double bond (Vogel et al. 2008). Other CCD1 substrates include ζ-carotene, lycopene, β-carotene, zeaxanthin, δ-carotene, and lutein.

Enzymatic assays with AtCCD1 show that it is able to cleave linear and cyclic carotenoids (Holger et al. 2006). *In vitro* cleavage of β-carotene results in formation of two C_{13} β-ionones, and a C_{14} dialdehyde. Detecting CCD1 substrates through *in vitro* enzymatic assays has been performed for a number of CCD1 orthologues. This process is, however, not straightforward as CCD1 is soluble in aqueous solutions and needs to interact with

hydrophobic carotenoids. To achieve a functional *in vitro* assay, a micellar system is typically used (Mathieu et al. 2007). To circumvent this problem *E. coli* strains able to accumulate different carotenoids are frequently transformed with the *CCD1* orthologue of interest. Functionality is determined either by a loss of colour (indicating catabolism of the carotenoid), and/or by detection of the volatile apocarotenoid products produced by the reaction.

CCD1 orthologues have been identified and, to varying extents, characterised in grape, petunia, coffee, crocus, citrus, tomato, melon, nectarine, rose, maize and star fruit. The native *in planta* function of CCD1 has proved challenging to determine. CCD1 is the only plant CCD that is not predicted to be targeted to the plastid, but is rather cytosolic (Vogel et al. 2008). This creates uncertainty as to how it accesses its plastidic carotenoid substrates. (Markwell et al. 1992) however, demonstrated that significant levels of β-carotene exist in the outer envelope of the pea chloroplast, which would be a possible source of substrate for CCD1 cleavage.

To date the *Arabidopsis* and tomato CCD1 orthologues exhibit the most substrate promiscuity (Vogel et al. 2008). Both are able to cleave ζ-carotene, lycopene, β-carotene, zeaxanthin, and δ-carotene. It is likely that orthologues from other species will have similar substrate promiscuity, as an amino acid sequence identity of approximately 80% exists between CCD1 plant orthologues (Vogel et al. 2008).

Tomato contains two copies of CCD1 (LeCDD1A and LeCCD1B). These orthologues catalyse the cleavage of a number of carotenoids *in vitro* generating a variety of volatile flavour compounds including geranylacetone, pseudoionone, β-ionone and 6-methyl-5-hepten-2-one (MHO) (Simkin et al. 2004a). Silencing *LeCCD1* in tomato fruit shed light on the *in planta* function of the gene, and led to reduced production of β-ionone and geranylacetone (a lycopene-derived apocarotenoid). Surprisingly there was no significant change in carotenoid concentrations in the tomato fruit (Simkin et al. 2004a).

Monitoring of native *CCD1* expression in tomato indicates that while *CCD1* is expressed in the leaf, expression is highest in the fruit. During ripening *LeCCD1* expression increases, while the production of volatiles through CCD1 enzymatic cleavage lag behind this increase in expression by about a week (Simkin et al. 2004a). The same phenomenon is seen in grape

berries, where *VvCCD1* expression peaks at véraison and the volatile apocarotenoids a week later (Mathieu et al. 2005). It is possible that the compartmentalisation of CCD1 and its substrates contributes to this phenomenon.

In *Arabidopsis*, *AtCCD1* expression does not correlate with the carotenoid content of leaves, but does correlate strongly with a reduction in the carotenoid content of maturing seeds (Holger et al. 2006). *Arabidopsis* plants possessing a non-functioning *AtCCD1* exhibit a higher concentration of carotenoids in mature seeds. It is thought that the rupturing of cellular organelles during seed drying allows for CCD1 to access its substrates. A similar process may occur in ripening fruit as carotenoid-containing chromoplasts differentiate and provide CCD1 access to its substrates. The high osmotic stress that occurs during fruit ripening may cause rupturing of cell membranes and result in exposure of carotenoids to CCD1 enzymes.

It has been reported that *Petunia hybrida CCD1* (*PhCCD1*) transcription in the flowers correlates with levels of the floral volatile, β-ionone (Simkin et al. 2004b). Additionally, suppressing *PhCCD1* expression led to a decrease in β-ionone production. The endogenous regulation of *PhCCD1*, and β-ionone emission, adheres to a circadian rhythm which has been suggested is an indication of its role as a pollinator attractant (Simkin et al. 2004b). An increase is observed in volatile emission during the day when potential insect pollinators are active. In rose *RdCCD1* expression was highest in flowers, and correlated with an increase in the production of C_{13} apocarotenoids (Huang et al. 2009).

In mycorrhizal roots of *Medicago truncatula* Floβ et al. (2008) postulate that the primary substrates for *MtCCD1* are C_{27} apocarotenoids produced from *MtCCD7* cleavage of carotenoids. Experiments that silenced *MtCCD1* expression in the roots of *M. truncatula* resulted in an increase in C_{27}-apocarotenoids (Floss et al. 2008). They suggest that MtCCD7 catalyses primary carotenoid cleavage in the plastid, and the C_{27} apocarotenoid products are subsequently transported to the cytosol where MtCCD1 is able to catalyse further cleavage.

It would appear that the most significant and observable effects of the cytosolic *CCD1* occur in the flowers or fruits of plants. These non-photosynthetic organs presumably exert a less stringent control on the carotenoid organisation, providing enzyme access to the carotenoid substrates.

2.4 Conclusions

CCD-derived apocarotenoids play a crucial biological role in plant metabolism, and are often of economic value. In plants they act as hormonal signalling molecules, such as ABA and strigolactone, where they perform crucial roles in the plant's growth and development. In fruits and flowers many apocarotenoids, such as β-ionone and geranylacetone, act as pollinator attractants, in this way facilitating reproduction. The CCD family also contributes to the maintenance of the plant's carotenoid content. This means that they are of importance when attempting metabolic engineering of carotenoids. While the biological role CCDs play in plants is beginning to be understood, there still remain many apocarotenoids the function of which is poorly understood. However, the genes involved in apocarotenoid synthesis are well characterised allowing for the investigation of apocarotenoid regulation and function through, amongst others, genetic manipulation.

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Chapter 3

Research results

The development of a method for the extraction of carotenoids and chlorophylls from grapevine leaves and berries for HPLC profiling

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The development of a method for the extraction of carotenoids and chlorophylls from grapevine leaves and berries for HPLC profiling

Justin G Lashbrooke, Philip R Young, Albert E Strever, Charmaine Stander, Melané A Vivier

3.1 Abstract

Background and Aims

Carotenoids and chlorophylls perform a number of essential roles in plants making their accurate quantification important to a variety of studies. We aimed to develop an extraction protocol to accurately determine the photosynthetic pigments in grapevine leaf and berry tissue, specifically focusing on limiting the degradation of these pigments.

Methods and Results

An extraction protocol for grapevine leaf and berry tissue was systematically optimised by identifying a number of critical parameters. Extracted pigments were analysed using Reversed Phase High Performance Liquid Chromatography (RP-HPLC). Specific parameters that were optimised included avoiding freeze-drying the material, the volume of acetone, the time required to extract all the pigments from the tissue, the addition of 0.1% (v/v) N-ethyldiisopropylamine to berry extracts to minimise pigment degradation during the extraction procedure, and avoidance of a concentration of the extracts which otherwise would result in differential degradation of pigments. Additionally, the method of extraction and normalisation with an internal standard was adapted and improved for accuracy. The optimised protocol was validated using authentic standards and its utility shown by analysing the pigment content of berries and leaves at different growth stages.

Conclusions

A method has been developed that is able to extract and accurately quantify, by means of HPLC profiling, the levels of photosynthetic pigments from grape berries and leaves. The method avoided any degradation of the pigments during the extraction and was applicable to both berries and leaves in different stages of growth and development, indicating its general

usefulness to vegetative and reproductive organs, even if their metabolic states are very different.

Significance of Study

The divergence of methods used for photosynthetic pigment analysis in plants, each with specific advantages and disadvantages were considered and used to optimise a number of parameters in a single method that proved to be applicable to plant organs in different developmental stages. The method is fast, applicable to vegetative and reproductive grapevine tissues, avoids degradation of pigments and ensures maximum accuracy when quantifying these important pigments.

3.2 Introduction

The well-studied role performed by chlorophylls and carotenoids during light harvesting is only one of a number of conserved functions these photosynthetic pigments perform in plants. In leaves (the primary photosynthetic organs) the carotenoids are associated with the chlorophylls and serve as both an accessory to the light harvesting pigments and as photoprotectants during high light conditions. The well characterised xanthophyll cycle (Demmig-Adams & Adams 1996) involves the reversible conversion of violaxanthin to zeaxanthin under high light conditions; enabling the dissipation of the excess absorbed energy as heat and the scavenging of reactive oxygen species (Figure 1).

Carotenoids also serve as precursors to the plant hormone abscisic acid (ABA), formed from the cleavage of neoxanthin (Figure 1) (Rock & Zeevaart 1991). The carotenoid biosynthetic pathway is regulated and highly sensitive to the influence of abiotic and biotic stress (Hugueney et al. 1996, Strzalka et al. 2003). The concentration of carotenoids and chlorophylls provides information about the level of stress the plant is experiencing as well as its ability to endure these stresses (Strzalka et al. 2003). The analysis of these photosynthetic pigments is therefore important in a number of studies.

In grapevine berries the concentration of carotenoids is of particular interest as they are known to be the pre-cursors of C_{13}-norisoprenoids (Figure 1) which contribute to the varietal flavour and aroma of wines (Baumes et al. 2002, Mendes-Pinto et al. 2009). The levels of the various carotenoids in grapes are influenced by a number of factors, including the cultivar, the exposure of grapes to sunlight and the degree of ripeness (Marais et al. 1991, Baumes et al. 2002, Oliveira et al. 2004).

The research on chlorophyll and carotenoid concentrations in plant organs requires that accurate profiling techniques exist so as to better understand the functions of genes and pathways being manipulated through transgenic or more classical experiments. A number of factors make the analysis of carotenoids a non-trivial process. Most of these factors are due to the intrinsic properties of the pigments themselves. They are hydrophobic and primarily soluble in organic solvents. The readiness of some organic solvents to evaporate at room temperature and the difficulties involved in pipetting such solvents creates a number of complications when maintaining constant concentrations of dissolved pigments. Photosynthetic pigments are also highly susceptible to degradation when exposed to light,

oxygen, aqueous conditions and high temperatures (Oliver & Palou 2000). This degradation is seen in structural and configuration modifications which alter the absorbance spectra and the chromatographic properties of the pigments (Ladislav et al. 2005). Due to its widespread occurrence during pigment extraction from grapevine, one degradation event of particular importance is the conversion of chlorophyll *a* to pheophytin *a* (Mendes-Pinto et al. 2005, Deluc et al. 2009). While there has been extensive investigation into the cause and prevention of possible detrimental effects to pigments during extraction procedures, the variability of the starting tissue and the particular aims of each protocol results in little consensus on what parameters are most critical. This is reflected in the multitude of techniques published with divergent methodologies. What is clear is that many published methods show high levels of degradation of chlorophyll (Mendes-Pinto et al. 2005, Deluc et al. 2009) and possibly some of the other extracted pigments.

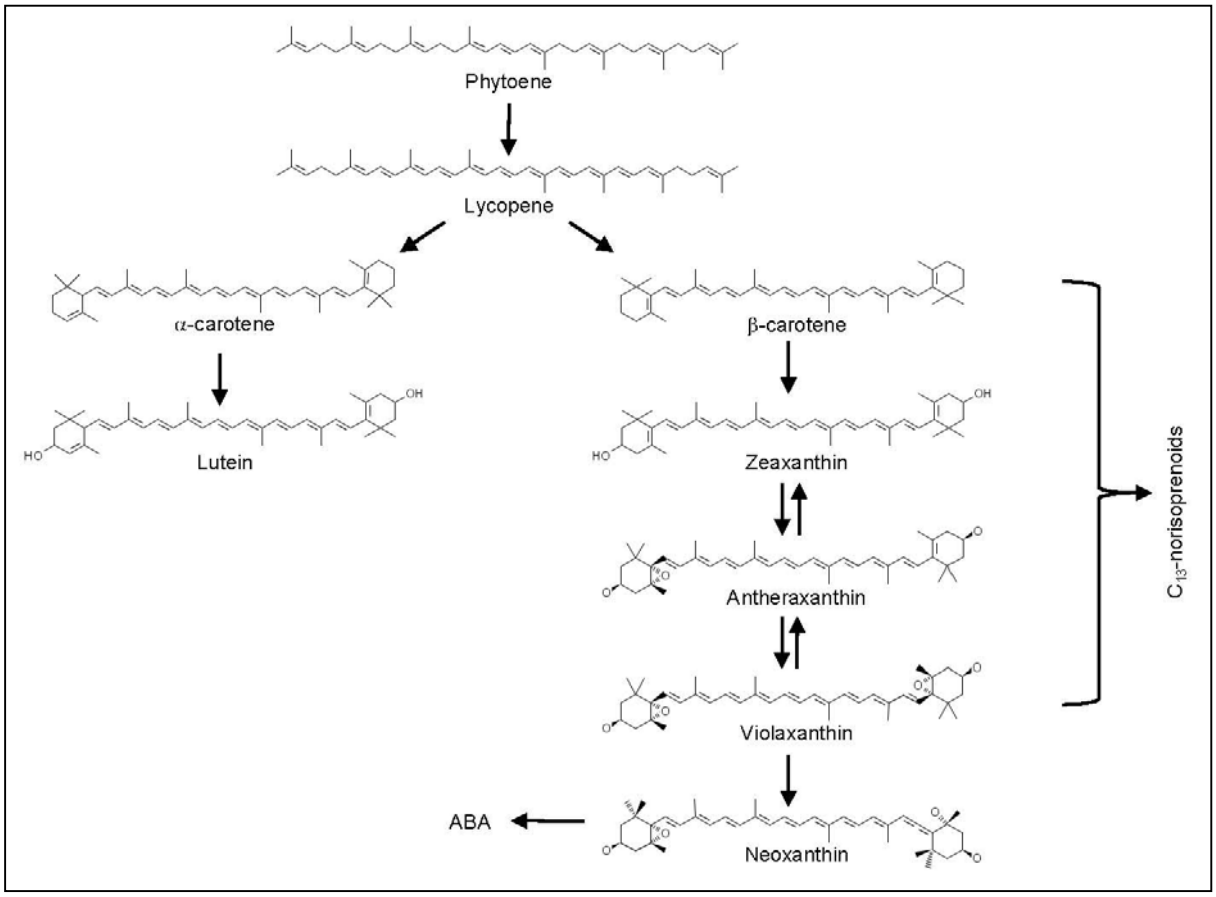

Figure 1. Schematic of the carotenoid pathway occurring in higher plants.

When developing a profiling technique it is necessary to consider the specific organism as well as the tissue type under investigation – the so called matrix effect. Previously an HPLC method to profile pigments from *Arabidopsis thaliana* leaf tissue was developed, which focused mostly on accurate separation of the carotenoids (Taylor et al. 2006), but significant problems with its application to pigment extraction from grapevine tissues was experienced. A systematic approach was used to optimise a method to extract chlorophyll and carotenoid pigments from grapevine berries and leaves suitable for HPLC profiling and quantification. Pigment degradation was successfully controlled with the method, while pigment loss during the extraction was monitored by the modified use of an internal standard. The utility of the optimised extraction protocol is illustrated in two experimental settings measuring the pigment concentration in developing grapevine organs.

3.3 Materials and Methods

3.3.1 Analytical solvents and chemicals

All solvents used during the sample extraction, preparation and analysis were of HPLC grade. *Tert*-butyl methyl ether (TBME), methanol, acetone, ethyl acetate and chloroform were purchased from Sigma–Aldrich (Steinheim, Germany). Butylhydroxytoluene (butylated hydroxytoluene, BHT), N-ethyldiisopropylamine (NED) and analytical grade NaCl were purchased from Fluka Chemie (Buchs, Switzerland). Triethylamine (TEA) and ammonium acetate were purchased from Merck (Hohenbrunn, Germany); Tris base was purchased from Roche Diagnostics (Mannheim, Germany). Pigment extraction buffer consisted of 50 mM Tris-HCl (pH 7.5) and 1 M NaCl.

3.3.2 Handling and preparation of authentic standards

The authentic standards *trans*-β-carotene, chlorophyll *b* and the internal standard (IS), β-apo-caroten-8-al, were purchased from Fluka Chemie. CaroteNature GmbH (Lupsingen, Switzerland) supplied zeaxanthin, neoxanthin, violaxanthin and antheraxanthin. Lutein and chlorophyll *a* were obtained from Sigma-Aldrich. See Table 1 for details of standards' purity and concentrations.

The standards were dissolved in chloroform containing 0.1% (w/v) BHT and divided into aliquots containing approximately 200 µg of pigment (depending on purity). The aliquots were then dried under a stream of N_2 (g) and stored at -20°C to prevent isomerisation (Lichtenthaler & Buschmann 2000, Ladislav et al. 2005).

Table 1. Details of authentic standards used, concentrations and wavelengths of quantification

Authentic Standards	Purity (%)	Working stock concentration ($\mu g.mL^{-1}$)	Wavelength of quantification (nm)
Antheraxanthin	95	1.90	450
β-apo-caroten-8-al	96	1.92	420
trans-β-carotene	97	3.84	450
Chlorophyll *a*	95	28.50	420
Chlorophyll *b*	95	19.0	470
Lutein	94	3.76	450
Neoxanthin	97	1.94	420
Violaxanthin	95	1.90	420
Zeaxanthin	97	1.94	450

3.3.3 Sample collection, storage and preservation

All tissue was immediately flash-frozen in liquid nitrogen after harvesting and stored at 80°C. The tissue was ground in liquid nitrogen to a fine visually homogenous powder. Seeds were removed from berry samples prior to grinding. During all experimentation care was taken to avoid tissue exposure to light and heat.

3.3.4 Plant material and characterisation

Optimisation experiments were performed on fully expanded leaf tissue, harvested from *V. vinifera* L. cv. Shiraz at the Welgevallen experimental farm (Stellenbosch University, South Africa) during the 2008-2009 growing season. A typical, non-topped shoot was chosen from a healthy vine one week after berry set occurred and leaves were sampled at regular intervals along the shoot from the base to the tip. Leaves were detached and leaf discs were collected from four inter-veinal areas on each leaf. The photosynthetic pigment content of the leaves was measured using the optimised protocol described below. The de-epoxidation state (DEPS) of the leaves was calculated as DEPS = (Z + 0.5A) / (V + A + Z) (Demmig-Adams & Adams 1996). The Chlorophyll a:b ratio (chlorophyll *a* / chlorophyll *b*) was determined for the different leaf positions.

V. vinifera L. cv. Dauphine grape berry tissue was obtained from Irene farm, (Paarl, South Africa) during the 2007-2008 growing season. Berry samples were collected at eight time points with 10 to 14 day intervals during the berry development period and analysed for sugar and acid content to profile the characteristic berry development and ripening phases. The photosynthetic pigment content was measured using the optimised extraction protocol described below.

Sugars and organic acids were extracted from the berries according to Broeckling et al. (2005). Approximately 1 g of frozen berry tissue was added to 10 mL chloroform and vortexed. The samples were incubated at 50˚C for 45 minutes. After equilibrating to room temperature, 10 mL water containing 20 mg ribitol (IS for the sugars) and 5 mg adipic acid (IS for the organic acids) was added. The sample was vortexed and incubated again for 45 minutes. The mixture was partitioned via centrifugation (3000 g, 30 minutes) at 4˚C. The upper aqueous phase was collected for analysis. The phenolic compounds present in post-véraison grapes were removed by adding 0.5 g polyvinylpyrrolidone (PVP) to 10 mL of the aqueous phase to prevent interference of these compounds with the organic acid analysis (Zotou et al. 2004). The mixture was stirred for 15 minutes, and the supernatant collected after centrifugation (1400 g, 15 minutes).

Major organic acids and carbohydrates present in the berries were analysed simultaneously using an HPLC system with refractive index (RI) and ultraviolet (UV) detection (Castellari et al. 2000). A Waters 717 auto-sampler, 410 refractive index detector and Agilent 1100 pump, controlled with Waters Empower software was used. An Aminex HPX-87H column (Bio-Rad Laboratories) was used to separate the compounds. Elution was performed with 5 mM H_2SO_4 at 45˚C with a flow rate of 0.5 mL.min^{-1}. In all cases 10 µL of the extract was injected.

3.3.5 Sequential extraction protocol

Ground leaf tissue (20 mg) was vortexed in 300 µL acetone containing 2 µg β-apo-caroten-8-al (IS) for 15 minutes. One mL pigment extraction buffer was added and the mixture vortexed briefly. Ethyl acetate (200 µL) was added and the mixture separated via centrifugation (10,000 x g) for 5 minutes. The upper ethyl acetate layer (containing pigments) was removed. A further 200 µL ethyl acetate was added and the tube was vortexed for 15 minutes. The pigments were separated into the ethyl acetate phase via centrifugation and removed. This was repeated three times so that four sequential ethyl acetate fractions were collected.

3.3.6 Single step extraction protocol

Frozen tissue from leaves (±20 mg) or berries (±250 mg) were extracted in 2.0 mL micro-centrifuge tubes in a cold laboratory (8°C), taking care to minimise exposure to air and light. The leaf samples were suspended in 1800 µL acetone and the berry samples in 1600 µL acetone containing 0.1% (v/v) NED. The internal standard, β-apo-caroten-8-al, was added to all extracts (2 µg). The tubes were vortexed for 30 minutes, followed by pelleting of the

tissue debris via centrifugation (10,000 g, 3 minutes). A 300 uL aliquot of the acetone extract (now containing pigment) was removed and added to 1 mL of extraction buffer and washed by vortexing for 5 minutes. Ethyl acetate (200 µL) was added, vortexed briefly followed by centrifugation (10,000 g, 5 minutes) to partition the mixture. A 50 µL aliquot of the 150-200 µL upper ethyl acetate phase (containing pigment) was removed, added to 200 µL methanol containing 0.125% (w/v) BHT, and 200 µL transferred to amber HPLC vials (containing 200 µL vial inserts) and sealed.

3.3.7 Protocol optimisations

Leaf tissue was ground and 20 mg samples weighed off. Samples were freeze-dried overnight while duplicate samples were stored at -80°C. Both sets of tissue were extracted according to the optimised protocol detailed above.

Ground leaf tissue (±20 mg) was extracted in either 900 µL or 1800 µL of acetone and ground berry tissue (±250 mg) in either 1200 µL or 1600 µL. The extractions then continued as described in the optimised method above.

Ground leaf tissue (±20 mg) extracted in 1800 µl acetone for 5, 10, 15, 20, 25, 30, 45 or 60 minutes. The extractions then proceeded as described in the optimised method above.

Ground leaf tissue (±20 mg) was extracted as described above modifying only the primary extraction solvent (1800 µL acetone) by adding either 0.1% (v/v) TEA or 0.1% (v/v) NED and compared to samples extracted without the additions.

Ground berry tissue (±250 mg) was extracted as described above modifying only the primary extraction solvent (1600 µL acetone) by adding 0.1% (v/v) TEA, 0.1% (v/v) NED, 0.2% (v/v) NED, 0.4% (v/v) NED, 0.04% (w/v) $CaCO_3$ or 0.08% (w/v) $CaCO_3$ and compared to samples extracted without the additions.

Pigments were extracted from leaf and berry tissue according to the optimised protocol above. Three 50 µL aliquots were removed from the ethyl acetate phase containing the pigments. One was evaporated till dryness under a stream of N_2 (g). A second 50 µL aliquot was evaporated till dryness in a DNA 120 Speed Vac concentrator (Savant, Minnesota, USA). Care was taken to prevent exposure of samples to light and high temperature

conditions during the drying step. The dried pigments were resuspended in 250 µL ethyl acetate, methanol (1:4) containing 0.1% (w/v) BHT. The third 50 µL fraction of ethyl acetate was added to 200 µL methanol containing 0.125% (w/v) BHT.

3.3.8 Chromatographic analysis of carotenoids and chlorophylls

All pigments were separated by RP-HPLC on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a DAD system. A YMC30 column (250 mm × 2.1 mm) and YMC30 guard cartridge (10 mm × 2.1 mm, particle size 3 µm) from YMC Europe (Schermbeck, Germany) were used. Chemstation software for LC3D (Rev.A.10.01[1635]; Hewlett-Packard, Waldbronn, Germany) was used for data processing.

The chromatographic conditions were modified from Taylor et al. (2006) to separate the major pigments extracted from grapevine tissue. A binary mobile phase of 3% (v/v) ddH_2O in methanol containing 0.2% (w/v) ammonium acetate and 0.05% (v/v) triethylamine (solvent A) and TBME containing 0.05% (v/v) triethylamine (solvent B) was employed. The flow rate was 1 mL.min^{-1} with the temperature maintained at 25°C. The elution program was isocratic at 20% B for 12 minutes followed by a linear increase from 20% to 50% B in 6 minutes, isocratic at 50% B for 4 minutes, a linear increase to 68% B in 2 minutes, isocratic at 68% for 4 minutes followed by a linear decrease to 20% B in 2 minutes. The column was equilibrated for 15 minutes at the starting conditions before each injection.

3.3.9 Identification, quantification and recovery of pigments

Pigments were identified by comparison of their retention times and spectral properties with the authentic standards and published data (Bramley 1992, Oliver and Palou 2000, Rodriguez-Amaya & Kimura 2004). Construction of external standard curves was performed by plotting amount (ng) against the area under the chromatogram at the relevant wavelength (Table 1). The curves were created by injection of 50 µL of serial dilutions of the working stocks (Table 1). The stocks were diluted in 1:4 ethyl acetate, methanol containing 0.1% (w/v) BHT to cover the range of pigment concentrations typically found in the extracts (80%, 60%, 40%, 20%, 10%, 5% and 2% of the working concentration). Standard curves were constructed for all the quantified pigments, except pheophytin *a,* which was quantified using the curve of chlorophyll *a*. The correlation co-efficient and regression equations were obtained using Statistica 8 software (Statsoft, Tulsa, UK). Due to the fact that only a fraction of the extract was injected and quantified on the column the concentration of the pigments

quantified was subsequently normalised to the concentration of the IS present in the injected sample.

Standard addition was performed to determine the recovery of pigments from both the leaf and berry single step extractions. Berry and leaf samples were spiked with authentic standards at the beginning of the extraction process (i.e. when acetone was added to the ground material). Duplicate samples were extracted without the addition of authentic standards to determine the endogenous level of pigments in the samples. The authentic standards were spiked at a concentration of approximately 50% of that occurring in the unspiked samples.

3.4 Results

The specific parameters identified in this study that were targeted for optimisation are summarised in Table 2.

3.4.1 Effect of freeze-drying material

Freeze-drying leaf material prior to extraction resulted in approximately 10% reduction of total pigments, with differential recoveries of the individual pigments observed (Figure 2). Chlorophyll *b* and antheraxanthin were the most severely affected with a reduction of 15% and 25% in recovery when compared to extracts from frozen tissue, respectively. β-carotene and chlorophyll *a* however were the least affected, showing a reduction in recovery of 10%.

3.4.2 Sequential extraction of pigments

Four sequential extractions were required to extract total pigments from 20 mg leaf tissue (Figure 3). The first, second, third and fourth sequential extractions contained 66%, 23%, 7% and 3% of the total pigments extracted, respectively. In contrast 79%, 18%, 3% and 0% of the IS was recovered.

The total amount of pigments extracted and quantified from 20 mg leaf tissue using the sequential extraction was compared to the total amount quantifiable when using the optimised single step extraction protocol. The single step extraction produced higher recoveries for the pigments of interest (Figure 4).

Table 2. Systematic optimisation steps

Steps in the extraction and separation procedure	Step optimised	Parameter optimised	Tissue type
Harvesting and preservation of samples	Sample storage	Effect of freeze-drying	Leaf
Extracting and preparing samples for analysis	Type of extraction	Sequential versus single step extraction	Leaf
	Total pigment extraction	Time of extraction	Leaf
		Volume of extraction	Leaf, Berry
	Prevention of pigment degradation	The use of additives to prevent degradation	Leaf, Berry
	Concentration	Effect of drying and concentrating on pigments	Leaf, Berry
Analysis of extracted pigments	Chromatographic conditions	Separation and quantification	Leaf, Berry

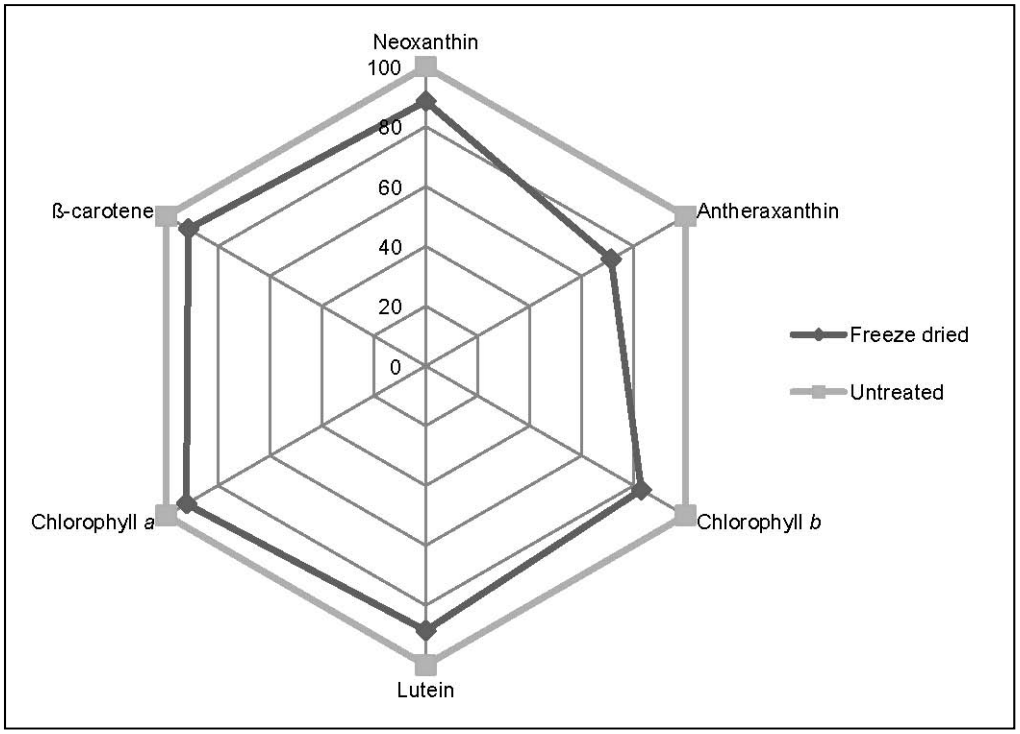

Figure 2. Effect of freeze-drying tissue. Pigments were extracted from 20 mg freeze-dried leaf material (◆) and compared to pigments extracted from 20 mg untreated material frozen (■).

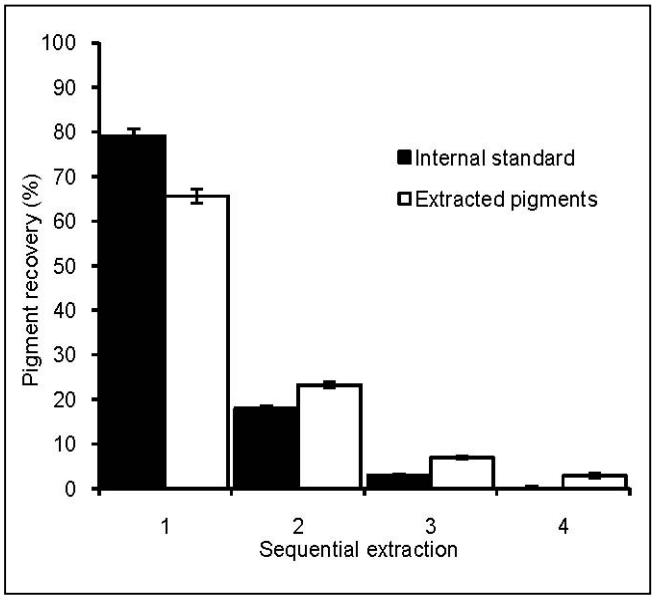

Figure 3. The differential recovery of the internal standard and the extracted pigments. The rate of recovery of the IS (■) to the extracted pigments (□) was measured during a typical sequential extraction protocol (n = 3).

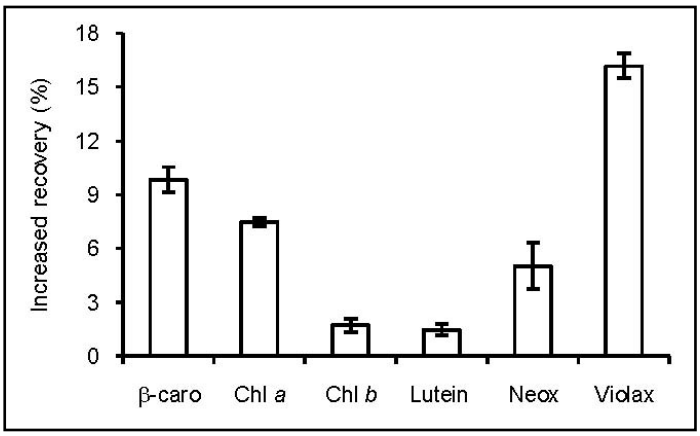

Figure 4. A single step extraction out performs a traditional sequential extraction. Duplicate tissue was extracted using either a single step extraction or a sequential extraction (n = 3). The results show the relative increase in recovery for the single step extraction (%). β-caro: β-carotene, Chl *a*: chlorophyll *a*, Chl *b*: chlorophyll *b*, Neox: neoxanthin, Violax: violaxanthin.

3.4.3 Extraction volume and time parameters

Extractions from 20 mg of leaf tissue in either 900 µL or 1800 µL acetone yielded a comparable amount of total pigments. Likewise pigments were equally extracted from 250 mg berry tissue using either 1200 µL or 1600 µL of acetone (data not shown). All further leaf and berry extractions were carried out using 1800 µL and 1600 µL acetone, respectively.

The majority (95%) of the total leaf pigments were extracted within the first five minutes of extraction (Figure 5). After 60 minutes no significant degradation was observed, but standard error increased. Subsequently it was decided to perform all further extractions for 30 minutes.

3.4.4 The effect of organic bases on pigment degradation

The addition of organic bases (0.1% (v/v) TEA or 0.1% (v/v) NED) reduced the total pigments that were extracted from leaf tissue by 20% (Figure 6). Extraction from berries, however, showed an improvement with the addition of 0.1% (v/v) NED (Figure 6 and 7). This improvement was evident in increase in chlorophyll *a* recovery (8%) and the reduction of pheophytin *a* (20%). $CaCO_3$ was detrimental to extractions causing a reduction of total pigment by 20% (Figure 6). TEA (0.1% (v/v)) was observed to be beneficial for the extraction of the carotenoids, but extracted 20% less chlorophylls. Additionally extracts containing TEA produced an unknown chlorophyll degradation product, putatively identified as chlorophyllide *a* (Figure 7B).

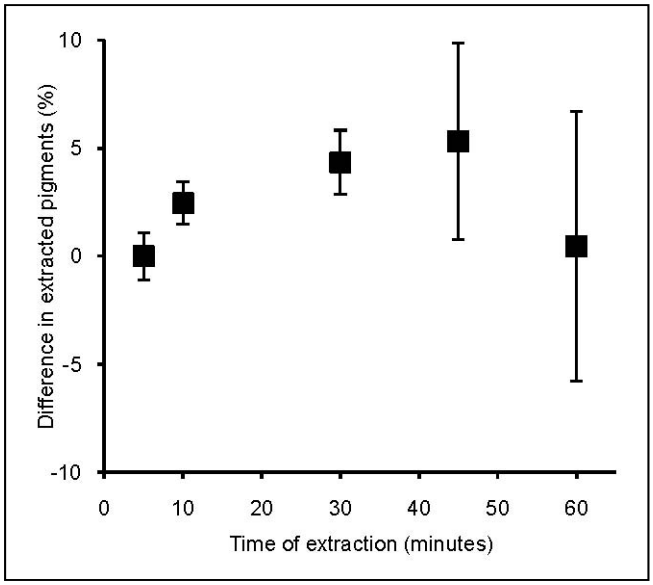

Figure 5. The difference in pigment recovery over time. Extractions were performed for various lengths of time on 20 mg of leaf tissue (n = 3). The percentage change in total pigments recovered is shown.

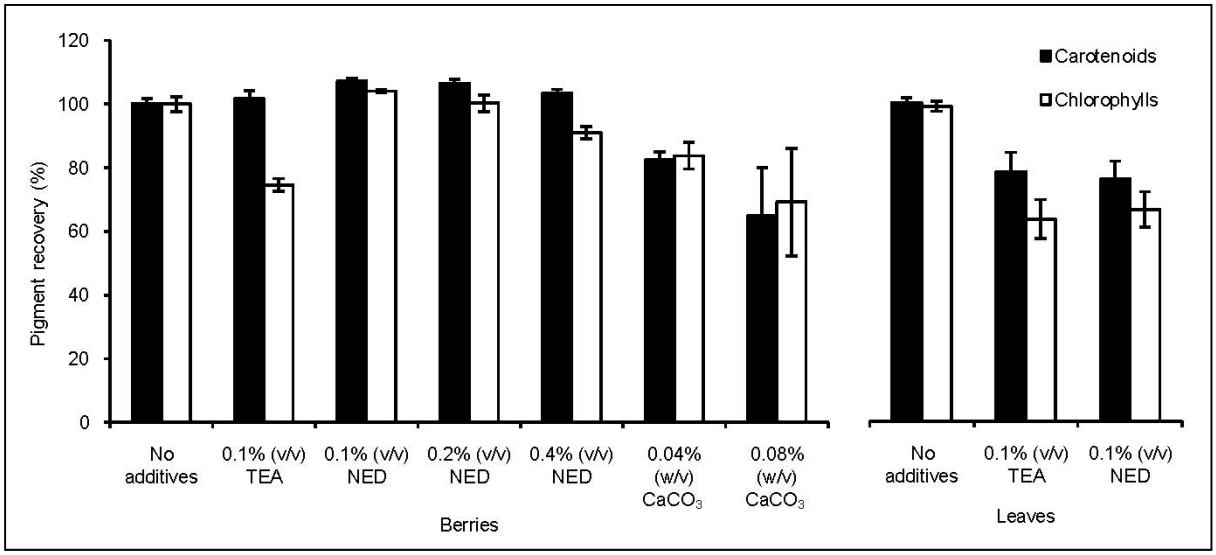

Figure 6. The effect of additives to the extractions from leaves and berries. Extracts were performed in acetone containing various additives (n = 3). The carotenoid (■) and chlorophyll (□) recoveries are expressed relative to a sample without additives.

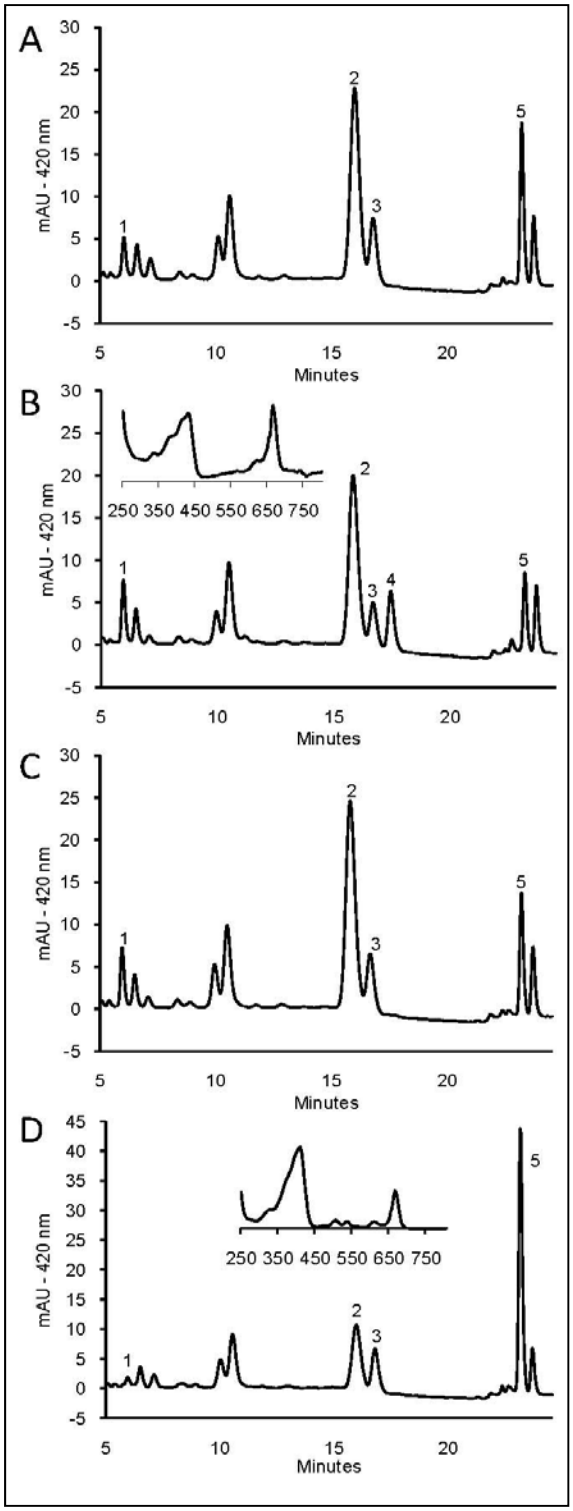

Figure 7. Chromatograms of berry extracts in the presence of various additives and after drying. An extract containing (A) no additives is compared to extracts containing (B) 0.1% (v/v) TEA and (C) 0.1% (v/v) NED. (D) The effect of drying the extracts using a Speedy-Vac is also shown. Peaks of interest include violaxanthin (1), chlorophyll *a* (2), β-apo-caroten-8-al (3), unknown chlorophyll (4), and pheophytin *a* (5). The absorbance spectra of the unknown chlorophyll degradation product is shown in the insert in B while the absorbance spectra of pheophytin *a* is shown in the insert in D.

3.4.5 Concentration of extracts

Drying extracts with a Speed Vac caused a significant amount of degradation of both carotenoids and chlorophylls (Figure 8). The degradation was not constant for all investigated pigments; violaxanthin and chlorophyll *a* totally degraded, whilst β-carotene and lutein showed no degradation. Drying under a stream of N_2 (g) resulted in less degradation; however violaxanthin levels were still reduced by 15% (Figure 8) relative to the non-concentrated samples. Both concentration methods resulted in the increase of pheophytin *a* formation (Figure 7D and 8).

Non-concentrated pigments in ethyl acetate were not directly injected onto the HPLC column due to incompatibility with the predominantly methanol mobile phase of the system. The ethyl acetate phase was combined with methanol containing 0.125% (w/v) BHT in a ratio of 1:4 so as to mimic the injection buffer used in Taylor et al. (2006). As seen in Figure 7 and 9 this lead to separation and well defined peaks on the chromatograms. It has been shown that incompatible injection solvents can cause poor separation and tailing of chromatogram peaks (Scott 1992).

3.4.6 Recoveries from the single step extraction protocol

Standard curves constructed for the authentic standards showed linearity over the concentration range injected. The r^2-values were above 0.9990 with the exception of violaxanthin (0.9989) (Table 3). Table 3 shows the recovery of spiked authentic standards from leaf and berry extracts. The recoveries are expressed relative to the recovery of the IS.

Table 3. r^2-values, retention times and recoveries from leaf and berry extracts for the relevant pigments

Compound	r^2	Retention time (mins)	Recovery from berry extracts (%)	Recovery from leaf extracts (%)
Antheraxanthin	0.9993	8.38	ND[a]	ND
β-apo-caroten-al (IS)	1.0000	15.57	NA[b]	NA
β-carotene	1.0000	23.86	100.8 ± 8.7	97.4 ± 10.1
Chlorophyll *a*	1.0000	16.56	119.8 ± 6.7	112.9 ± 11.7
Chlorophyll *b*	0.9991	9.85	93.9 ± 8.3	115.1 ± 9.7
Lutein	0.9998	10.42	91.7 ± 7.9	100.4 ± 9.8
Neoxanthin	0.9996	6.51	ND	102.8 ± 14.0
Violaxanthin	0.9989	5.95	105.9 ± 9.1	80.5 ± 9.3
Zeaxanthin	0.9999	12.85	89.1 ± 6.1	113.9 ± 3.8

[a] *Not Determined*
[b] *Not Applicable*

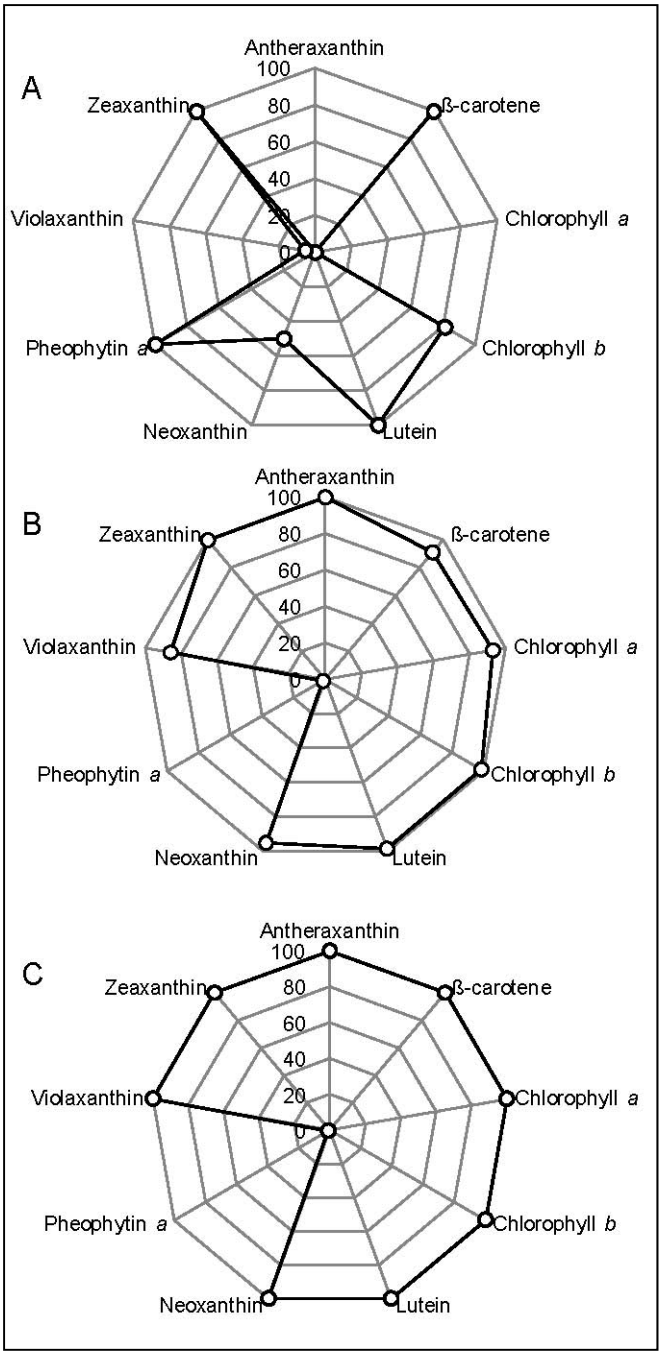

Figure 8. The effect of concentrating extracts Concentrating the extracts using a Speed-Vac (A) or under a stream of N_2 (g) (B) prior to injection was compared with directly injecting the extract (C).

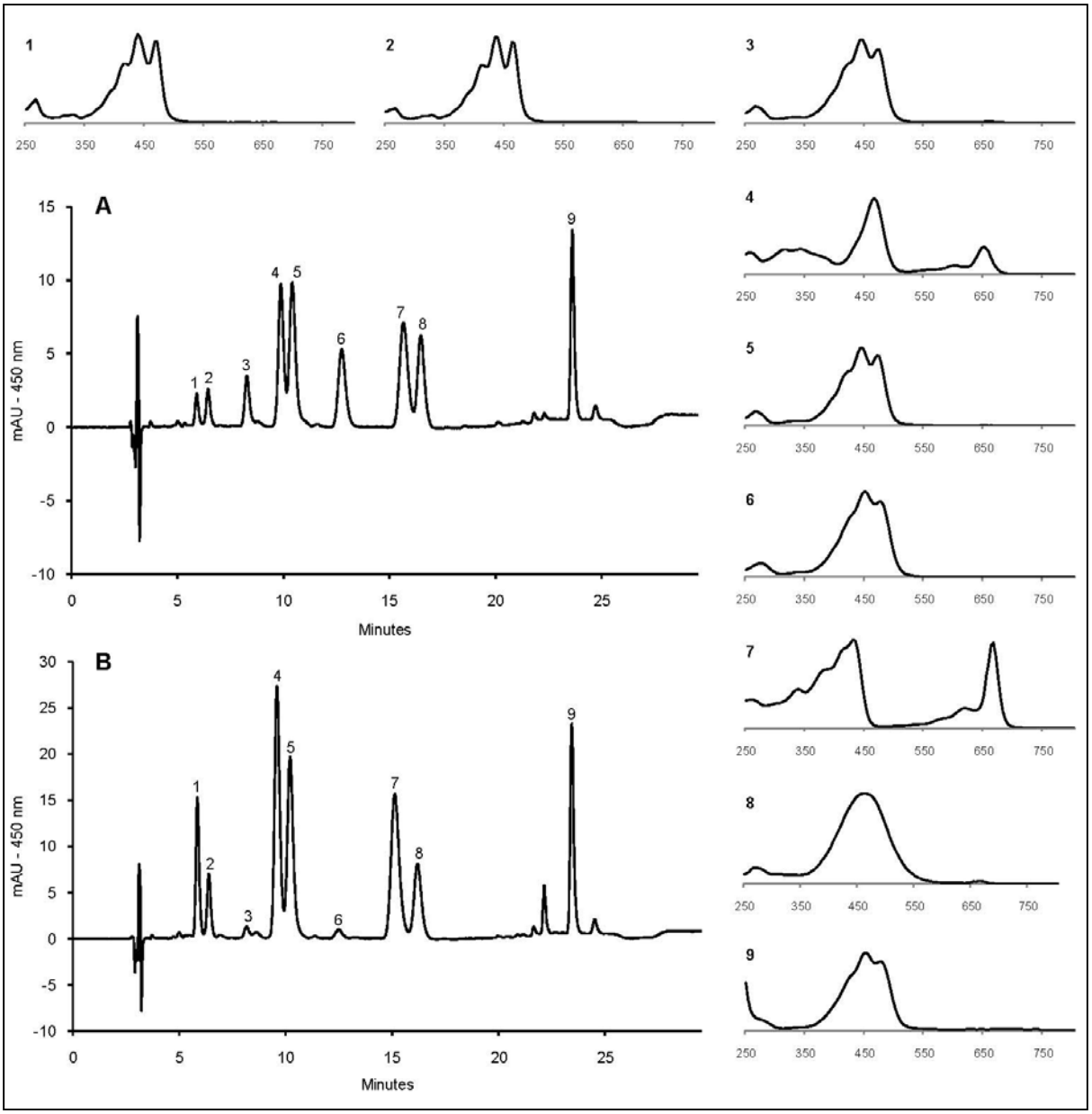

Figure 9 - Chromatograms of a young and a mature grapevine leaf. Extracts from (A) a young (node 16) and (B) a mature leaf (node 6) are shown. Both leaves are fully expanded and photosynthetically active. Peaks of interest are labelled and their respective spectra shown: violaxanthin (1), neoxanthin (2), antheraxanthin (3), chlorophyll *b* (4), lutein (5), zeaxanthin (6), chlorophyll *a* (7), β-apo-caroten-8-al (8), and trans-β-carotene (9). The absolute concentrations of the measured pigments are shown in Table 4.

3.4.7 Pigment levels in developing grapevine leaves and berries

The progress of berry development and ripening was monitored by analysing the sugar and acid concentrations and berry diameter (Figure 10A). Based on the decrease in acid concentrations and increase in sugars, véraison was determined to correspond to the period 8

weeks post-anthesis. The chlorophyll and carotenoid levels measured in the berries throughout ripening are shown in Figure 10B and 10C, respectively.

Similarly, the development of photosynthetic pigments in leaves from a typical grapevine shoot was analysed. The DEPS and the chlorophyll a:b ratio of the grapevine shoot is shown in Figure 11. Both the DEPS and the chlorophyll a:b ratio show an inverse correlation with leaf age.

Chromatograms of the extracts from a mature leaf (node 6) and a relatively young leaf (node 16) are shown in Figure 8. Both the leaves are fully expanded and photosynthetically active. The total pigment concentration measured in the two leaves is shown in Table 4.

Table 4. Comparison of pigment concentrations found in a mature leaf (node 6) and a young leaf (node 16). Both leaves are fully expanded and photosynthetically active. Pigment concentration is shown in $ng.mg^{-1}$ DW (n = 3).

	Antheraxanthin	β-carotene	Chlorophyll *a*	Chlorophyll *b*	Lutein	Neoxanthin	Violaxanthin	Zeaxanthin
Node 6	42.3 ± 0.7	417.2 ± 11.0	6959.8 ± 182.2	2760.6 ± 61.1	580.9 ± 19.6	315.9 ± 16.8	327.6 ± 10.7	34.0 ± 1.0
Node 16	136.9 ± 4.6	294.6 ± 3.0	4081.6 ± 37.2	1284.1 ± 1.0	356.4 ± 3.2	150.2 ± 0.3	70.5 ± 1.4	271.0 ± 6.7

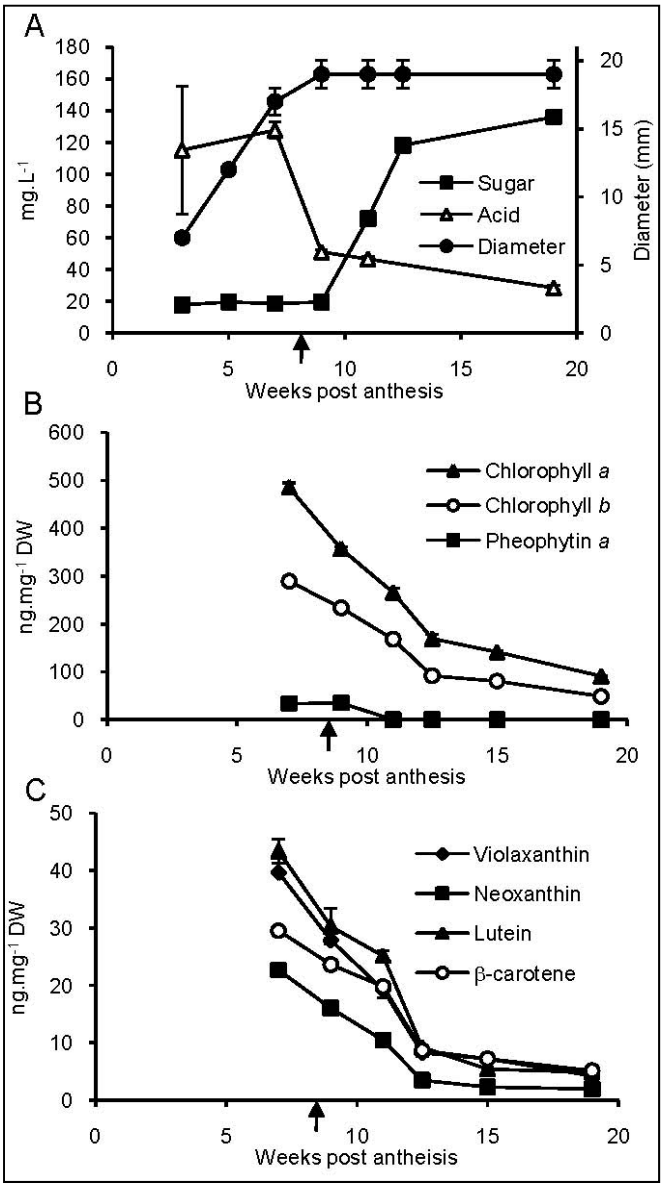

Figure 10. Characterisation of ripening *Vitis vinifera* L. cv. Dauphine berries. (A) The ripening of the berries was monitored by analysing the sugar (■) and acid (△) concentrations and the berry diameter (●) (n = 2). (B) The chlorophyll *a* (▲), chlorophyll *b* (O) and pheophytin a (■) concentrations, and (C) the violaxanthin (◆), neoxanthin (■), lutein (▲) and β-carotene (O) concentrations of the berries was analysed using the optimised single step extraction protocol (n = 3) Véraison is indicated on the graphs (↑).

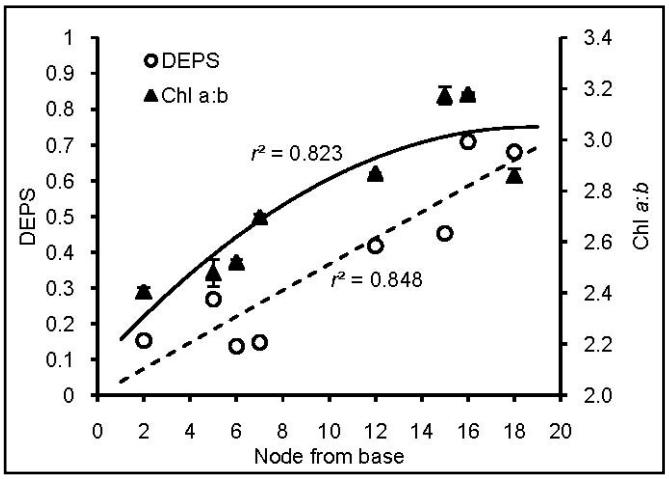

Figure 11. The DEPS and Chl a:b ratio of leaves on a typical grapevine shoot. Pigments were extracted using the optimised single step protocol (n = 3). The DEPS ratio (O) and Chl a:b ratio (▲) are shown.

3.5 Discussion

3.5.1 General considerations

Experiments were focused on the optimisation of sample preservation and the optimal extraction of pigments from the grapevine tissues. Through systematic steps (outlined in Table 2) these optimisations were applied to both grapevine leaves and berries in different developmental stages. Optimisations were aimed at improving both accuracy of pigment determination and efficiency of the extraction process, while attempting to minimise degradation occurring throughout the procedure.

Acetone has been shown to efficiently extract the photosynthetic pigments from a range of plant tissue types and organisms (Thayer and Bjorkman 1992, Dunn et al. 2004, Hendrickson et al. 2004), and considering that photosynthetic pigments are stable in this solvent (Dunn et al. 2004), it was selected as the primary extraction solvent used in this method. Instead of using ddH_20 (Hendrickson et al. 2004) as the aqueous phase to partition the pigments into the final solvent (ethyl acetate), the extraction buffer (50 mM Tris-HCl (pH 7.5) containing 1 M NaCl) was used (Fraser et al. 2000). The buffering capacity and high salt concentration of the buffer provides protection against both enzymatic and acid-induced degradation. β-Apo-caroten-8-al was used as an IS as it fulfilled certain criteria, such as possessing similar properties to the compounds under investigation while not occurring naturally in the tissue being investigated, and not co-eluting with any other extracted pigments. This IS has been

used previously in a number of studies (Olivera et al. 2004, Taylor et al. 2006). Extracted samples placed in sealed HPLC vials were observed to be stable for up to 24 hours when stored at -20°C (data not shown).

C_{18} and C_{30} HPLC columns have been shown to effectively separate photosynthetic pigments in a number of applications; C_{30} columns however have the added advantage of better separation of the more non-polar pigments (xanthophylls) (Emenhiser et al. 1995, Sander et al. 2000). Pigments were therefore separated on a C_{30} column using a reverse-phase HPLC system and identified and quantified spectrally with a photodiode array (PDA) detector.

3.5.2 Freeze-drying tissue degraded pigments

General sample storage and treatment is important when analysing pigments because their inherent properties make them highly susceptible to degradation by light, heat and oxygen (Oliver & Palou 2000). To determine whether freeze-drying samples would allow for greater stability of pigments, leaf samples were freeze-dried before extraction and compared to non freeze-dried tissue via RP-HPLC analysis. It is not clear if the reduction in pigment recovery observed (Figure 2) is due to degradation of the pigments or due to lower extraction efficiency from the freeze-dried tissue or both.

The advantages gained from freeze-drying (such as non-specialised storage and ease of handling) have resulted in this technique being commonly used during sample preparation (Cinar 2004, Rodriguez-Amaya & Kimura 2004). However, despite the advantages, the negative effect observed on pigment recoveries excluded its further use.

3.5.3 Sequential extraction can lead to inaccuracies during normalisation

The differential rate of recovery of the endogenous pigments and IS that was observed when performing a traditional sequential extraction stems from the fact that the IS is added directly to the extraction solvent and requires no extraction from the tissue matrix itself. For an IS to be used for normalisation during an extraction it is necessary that it remains at a constant ratio to the pigments under investigation, as any loss of the IS (through both degradation and pipetting error) is assumed to be proportional to the loss of the extracted pigments. The data generated (Figure 3) shows that the ratio of IS to extracted pigments is not constant. Losses from the first sequential extraction (with a ratio of 1.2) would therefore be overrepresented while losses from the subsequent extractions would be underrepresented. It is flawed to

assume that these overrepresented and underrepresented losses will simply cancel each other out. Assuming total extraction of the pigments from the tissue, a single step extraction would result in a constant ratio of IS to the extracted pigments and would therefore allow for accurate normalisation of all losses. A number of further optimisations of the single step extraction protocol were therefore concerned with confirming total pigment extraction from the tissue.

3.5.4 Optimised extraction volume and time parameters

During both berry and leaf extractions the larger volume of acetone experimented with was chosen for the primary extraction solvent so as to prevent any possible saturation of the solvent by the extracted pigments. Co-extracted compounds such as organic acids have been shown to cause acidification and destruction of the pigments (Lichtenthaler & Buschmann 2000, Rodriguez-Amaya & Kimura 2004). A larger extraction volume dilutes both the pigments extracted and more importantly the contaminants, reducing potential degradation. Despite the dilution of the pigments the final concentration injected was still in the quantifiable range.

Negligible differences in total pigments extracted were observed between 10 and 60 minutes (Figure 5), therefore slight deviations in the time of extractions would have no significant effect on the total pigments extracted. It was assumed that the chosen extraction time and volume was sufficient to extract all pigments from the tissue since an increase in extraction volume and duration of extraction showed no increase in total pigments extracted.

3.5.5 The use of organic bases to minimise degradation

Plant vacuoles contain high levels of organic acid which are liberated when tissue is ground during pigment extraction. Unripe grape berries have particularly high acid concentrations in their vacuoles (Conde et al. 2007) explaining the greater effect of alkaline additives on berry extracts when compared to leaf extracts. Previous attempts to neutralise these acids have made use of $MgCO_3$ and $CaCO_3$ (Cano 1991, Razungles et al. 1996, Lichtenthaler & Buschmann 2000) and the organic base NED (Kranner et al. 2003). The optimisation results indicate that these additives can prevent pigment degradation, but often are detrimental to the actual extraction process (Figure 6). It is possible that alkaline conditions are not suitable for the liberation of pigments from grapevine tissue.

Pheophytin *a* is a chlorophyll *a* molecule lacking the central Mg^{2+} ion. The conversion of chlorophyll to pheophytin is primarily caused by enzymatic action, acid hydrolysis and high temperatures (Park et al. 1973, Lichtenthaler & Buschmann 2000, Park et al. 2007). The presence of pheophytin *a* in chromatograms therefore, can often be regarded as a good indication that acid-induced pigment degradation has occurred. Because of the direct relationship between chlorophyll levels and pheophytin levels its presence in chromatograms is often overlooked and simply included in chlorophyll calculations. This however is an error prone approach as conditions that lead to chlorophyll degradation are generally the same conditions that could lead to the possible co-degradation of xanthophylls and carotenes. Carotenoid-derived degradation products are often not visible on the chromatogram making accounting for the loss of these pigments impossible. It was observed that violaxanthin degradation occurred in the same extracts as those with pheophytin *a* formation (Figure 7). This is in agreement with other studies where pheophytin *a* formation correlated with a reduction in violaxanthin (Esteban et al. 2009). The optimised single step extraction protocol however, shows very low levels of pheophytin *a* from leaf extractions without the need for any additives; while the use of 0.1% (w/v) NED in the primary extract during berry extractions was sufficient to minimise degradation, without being detrimental to the extraction of the pigments themselves.

3.5.6 Concentrating extracts leads to pigment degradation

Many extraction protocols employ a concentrating step after extraction of the pigments to increase the concentration of pigments injected onto the column (Rodriguez-Amaya & and Kimura 2004). HPLC analysis was performed to compare the concentration of pigments from samples that had been evaporated with those from non-evaporated samples.

The results indicated that two widely used methods for the concentration of extracts led to degradation of pigments (Figure 8). This is in accordance with observations made in other studies (Tonucci et al. 1995). Temperature control during the concentration step is often difficult and depending on the volume of the extract this can result in the pigments being at relatively high temperatures for up to 30 minutes. It is also important to realise that when concentrating the pigments any impurities in the extract will also be concentrated. This can cause an increase in acid concentration in the extract which in turn will lead to degradation.

Without concentration the amount of pigments extracted from 20 mg leaf tissue or 250 mg berry tissue was within the quantifiable range of detection. Direct injection avoided the degradation associated with concentration and allowed for a more rapid extraction protocol.

3.5.7 The optimised single step extraction protocol yielded high recoveries

The recoveries are expressed relative to the recovery of the IS (Table 3), which explains values being above and below 100% recovered. The 112% recovery of chlorophyll *a* for instance shows the pigment to be less susceptible to degradation or loss than the IS during extraction from leaf tissue. This illustrates the inherent difficulty when choosing a single IS to represent multiple compounds.

Importantly, while pheophytin *a* was detected in unspiked berry samples, there was no detectable increase in pheophytin *a* in the chlorophyll *a* spiked samples. This shows that the single step extraction method does not cause a conversion of chlorophyll *a* to pheophytin *a*, and that all measured pheophytin *a* originates either from the berry samples or the handling of the samples prior to extraction. This further illustrates the inaccuracy of including pheophytin levels in chlorophyll calculations as pheophytin *a* appears to be present naturally in berries and not formed exclusively as an extraction artefact.

3.5.8 Application of the optimised single step protocol

The optimised pigment extraction protocol was used to analyse the pigment content of grapevine leaves and berries in two typical situations to establish the utility of the method. In berries, a steady decline in the abundance of the chlorophylls and carotenoids after véraison is observed (Figure 10). This is in accordance with a number of previous studies (Razungles et al. 1996, Baumes et al. 2002). A decrease in pigment concentrations has been shown to correlate with an increase in the aromatic C_{13}-norisoprenoids. The carotenoid content of the berries therefore has a direct influence on the flavour of wines (Baumes et al. 2002). The successful implementation of the optimised extraction protocol to berries in different stages of development illustrates the usefulness of the proposed method.

In leaves an inverse correlation between the DEPS and the chlorophyll a:b ratio was observed (Figure 11). Chlorophyll *a* levels have previously been shown to be lower in both older (Hunter & Visser 1989, Bertamini & Nedunchezhian 2002) and more shaded leaves (Bjorkman & Holmgren 1963, Bertamini & Nedunchezhian 2004). Higher chlorophyll a:b

ratios correlate to increased photosynthetic activity in the younger more exposed leaves (Lichtenthaler et al. 1981). These leaves generally exhibit a higher DEPS value indicative of a shift in the xanthophyll cycle to zeaxanthin resulting in increased photoprotection. The utility of the proposed protocol is once again illustrated in the successful extraction and quantification of pigments from leaves differing in age and physiological and biochemical function.

3.5.9 Concluding remarks

We present not only a method for the extraction of photosynthetic pigments from grapevine tissue, but a detailed description of method optimisation. The protocol was vigorously and systematically optimised for all steps of the extraction process. Care was taken at all stages to identify and subsequently minimise conditions leading to degradation of the pigments, while simultaneously improving accuracy. This is specifically evident in the reduction of the conversion of chlorophyll *a* to pheophytin *a*, and the development of a single step extraction protocol. The chromatograms generated by this method display peaks that are well separated and easily quantified.

Due to the vast variety of plant metabolites (and their properties) it is unrealistic to hope for a single extraction protocol that will be capable of distinguishing and quantifying the majority of the metabolites found in a plant. The utility of this method however, is demonstrated in the efficacy, reproducibility and accuracy of pigment extraction and subsequent quantification from tissue types differing in age, developmental stage and organ. The use of this protocol to extract pigments from other tissues is yet to be investigated, although it is conceivable that with minor modifications this would be possible.

3.6 Acknowledgements

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Chapter 4

Research results

Functional characterisation of a *Vitis vinifera* carotenoid cleavage dioxygenase, *VvCCD1*

Functional characterisation of a *Vitis vinifera* carotenoid cleavage dioxygenase, *VvCCD1*

Justin G. Lashbrooke, Philip R. Young and Melané A. Vivier

4.1 Abstract

In plants carotenoids not only perform a crucial role during photosynthesis, but also serve as the precursors to apocarotenoids, a group of compounds with diverse biological functions. Enzymatic cleavage of carotenoids by CCD1 enzymes has been shown to produce a number of volatile flavour and aroma apocarotenoids including β-ionone, geranylacetone, pseudoionone, α-ionone and 3-hydroxy-β-ionone. In grapevine (*Vitis vinifera* L.) the pathways leading to important quality impact factors such as flavour and aroma are extensively studied. A CCD1 orthologue in grapevine (VvCCD1) has previously been observed to catalyse the *in vitro* cleavage of zeaxanthin and lutein. We were able to show that VvCCD1 is also able to catalyse the cleavage of lycopene at the 5,6 (5',6') and 9,10 (9',10') double bonds and β-carotene at the 9,10 (9',10') double bonds. In order to elucidate the *in planta* function of *VvCCD1 V. vinifera* lines were transgenically altered for *CCD1* expression through RNAi-mediated gene silencing and overexpression of the gene. A transgenic population showing a 12-fold difference in *VvCCD1* was generated and metabolically characterised. Photosynthetic tissue from these lines was analysed for carotenoid and apocarotenoid content via HPLC and GC/MS, respectively. Results indicate that a large amount of control is exerted on VvCCD1 activity, both post-transcriptionally and possibly by means of subcellular compartmentalisation. Since weak correlation between gene expression and apocarotenoid production was observed, it appears that the *in planta* action of VvCCD1, in photosynthetic tissue, appears to be distinct from the demonstrated *in vitro* activity. Carotenoid levels were more strongly correlated with chlorophyll concentrations than *VvCCD1* expression levels indicating that carotenoid biosynthesis is tightly regulated in favour of photosynthetic activity.

4.2 Introduction

Carotenoids are yellow, orange and red C_{40} isoprenoid pigments occuring in plants and microbes. In higher plants their primary role is light harvesting and photoprotection during photosynthesis. They are able to quench free radicals preventing photooxidative damage to the cell. The genes and enzymes involved in carotenoid biosynthesis in plants have received much attention and the conserved pathway is well characterised (Britton 1995, Cunningham 2002). Carotenoid biosynthesis occurs in the plastid as does the precursory methylerythritol phosphate pathway which produces isopentenyl diphosphate, the C_5 building block of carotenoids (Cunningham 2002). Due to their major role played during photosynthesis carotenoids are tightly regulated in photosynthetic organs. This is evident in the conserved ratio seen between the major carotenoids in plant photosynthetic tissue throughout nature (Liotenberg et al 1999). The carotenoid profiles seen in reproductive organs such as flowers and fruits however, are much more diverse (Tanaka et al. 2008).

The carotenoid cleavage dioxgenase (CCD) enzyme family, so named because of their ability to cleave carotenoids, contribute to the directed production of apocarotenoids. These apocarotenoids and their derived metabolites have diverse biological roles (Auldridge et al. 2006a). In plants they are signalling molecules, including the hormone-like strigolactone, and the phytohormone, abscisic acid (ABA); and contribute to the flavour and aroma of flowers and fruit; while industrially they form the basis for the colourants and spices in saffron and bixin. In *Arabidopsis thaliana* nine different CCDs have been identified and are named according to their discovery in this species (Auldridge et al. 2006b). Five of these enzymes are highly similar and cleave the 11,12 double bond of 9-*cis*- epoxycarotenoid substrates (e.g. neoxanthin and violaxanthin). These enzymes are NCED2, NCED3, NCED5, NCED6, and NCED9. The cleavage reaction they catalyse is the first step towards ABA synthesis (Chernys & Zeevaart 2000). The remaining four enzymes (CCD1, CCD4, CCD7 and CCD8) have more divergent roles and cleave a variety of carotenoid substrates at specific double bonds positions. Specifically, CCD7 and CCD8 cleavage result in the formation of the shoot-branching inhibiting hormone, strigolactone (Dun et al. 2009). CCD4 cleavage results in the production of a variety of flavour and aroma compounds (Huang et al. 2009).

CCD1 orthologues are highly homologous and have been shown to symmetrically cleave a variety of carotenoids *in vitro* at the 9,10 (9',10') double bond position producing a wide range of volatile C_{13} norisoprenoids including geranylacetone, pseudoionone, β-ionone, α-

ionone and 3-hydroxy-β-ionone (Simkin et al. 2004, Vogel et al. 2008) (Table 1). The tomato homologue (LeCCD1) has also been shown to cleave lycopene *in vitro* at the 5,6 (5',6') double bond forming 6-methyl-5-hepten-2-one (MHO) (Vogel et al. 2008). These carotenoid-derived C_{13} norisoprenoids are found in flowers, fruits, and leaves of many plants (Baldermann et al. 2005, Cooper et al. 2009). They are considered to be flavour and aroma compounds, often displaying very low detection thresholds. Due to their influence on the fruity and floral characteristics of wine C_{13} norisoprenoids have received interest in grapevine (*Vitis vinifera* L.) berries (Baumes et al. 2002, Mendes-Pinto 2009).

Grapevine CCD1 (VvCCD1) has specifically been shown to cleave lutein and zeaxanthin *in vitro* producing the volatile flavour compound 3-hydroxy-β-ionone (Mathieu et al. 2005, Mathieu et al. 2007). Gene expression analysis of *VvCCD1* in ripening berries showed an increase in expression levels at véraison, while an increase in volatile norisoprenoids, however, was only observed a week after véraison, raising questions about the *in planta* function of *CCD1* (Mathieu et al. 2005).

The current work aims to elucidate the biological role of *VvCCD1* in grapevine via over-expression and silencing strategies in grapevine. Previous attempts at transgenic manipulation of *CCD1* transcript levels in plants have been performed in *Lycopersicum esculentum* (tomato) (Simkin et al. 2004) and *Medicago truncatula* (Floβ et al. 2008). Both these studies generated results that suggested that the observed *in vitro* functioning of CCD1 (symmetrical 9,10 (9',10') cleavage of carotenoids) may not be its sole biological action *in planta*. A population of grapevine (*V. vinifera* L. cv. Sultana) transgenics with altered expression of *VvCCD1* was generated and genetically and phenotypically characterised. For this study all data was generated from the leaf tissue of the transgenic lines and analysed via HPLC and GC/MS for the detection and quantification of carotenoids and norisoprenoids, respectively.

Table 1. Characterisation of CCD1 orthologues.

Organism	% Identity	Accession	Substrate(s) tested	Reference
Vitis vinifera	100.0	AAX48772	zeaxanthin, lutein	Mathieu et al. 2005
Cucumis melo	83.6	ABB82946	phytoene, lycopene, δ-carotene, β-carotene	Ibdah et al. 2006
Citrus unshiu	86.8	BAE92957	β-cryptoxanthin, zeaxanthin, *trans*-violaxanthin, 9-*cis*-violaxanthin	Kato et al. 2006
Citrus sinensis	86.6	BAE92958	β-cryptoxanthin, zeaxanthin, *trans*-violaxanthin, 9-*cis*-violaxanthin	Kato et al. 2006
Phaseolus vulgaris	82.1	AAK38744	zeaxanthin	Schwartz et al. 2001
Arabidopsis thaliana	81.4	CAA06712	β-carotene, zeaxanthin, lutein, *trans*-violaxanthin, 9-*cis*-violaxanthin, 9-*cis*-neoxanthin	Schwartz et al. 2001
Petunia x hybrida	81.9	AAT68189	β-carotene	Simkin et al. 2004
Lycopersicon esculentum	81.9	AAT68187	*trans*-violaxanthin, 9-*cis*-neoxanthin, β-carotene, zeaxanthin, lutein, lycopene	Simkin et al. 2004
Crocus sativus	80.6	CAC79592	zeaxanthin	Bouvier et al. 2003
Coffea arabica	83.8	ABA43904	lycopene, β-carotene, zeaxanthin	Simkin et al. 2008
Zea mays	77.1	ABF85668	lycopene, β-carotene, zeaxanthin	Sun et al. 2008
Coffea canephora	84.1	ABA43900	lycopene, β-carotene, zeaxanthin	Simkin et al. 2008

4.3 Materials and Methods

4.3.1 In silico analyses

The National Center for Biotechnology Information (NCBI) Entrez search and retrieval system was used to obtain nucleotide and protein sequences from the Genbank databases (http://www.ncbi.nlm.nih.gov/Entrez/). Alignments to sequences in the Genbank databases were performed using the relevant Blast algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Comparative genomics (gene structure prediction and homologue/orthologue retrieval) were performed via Plaza (http://bioinformatics.psb.ugent.be/plaza/).

The putative sub-cellular localisation of protein sequences were predicted using ProtComp Version 8.0 (http://www.softberry.com/berry.phtml). *V. vinifera* expressed sequence tags (ESTs) were retrieved from The Institute for Genomic Research (TIGR) Grape Gene Index (http://compbio.dfci.harvard.edu/tgi/) or NCBI. The *V. vinifera* genomic sequences were retrieved from NCBI or Genoscope (http://www.cns.fr/externe/GenomeBrowser/Vitis/).

4.3.2 Isolation, extraction and manipulations of nucleic acids

High molecular weight genomic DNA was isolated from fully expanded *V. vinifera* leaves as described by Steenkamp et al. (1994). Total RNA from different grapevine leaves was extracted according to the methods described by Reid et al. (2006). Unless otherwise stated, all standard methods for plasmid DNA isolation, manipulations and cloning of DNA fragments, and agarose gel electrophoresis were used as described by (Sambrook, et al. 1989).

Total cDNA was synthesised from 1 µg RNA (after DNase I treatment (Promega, Madison, WI)) using the Superscript III Platinum first strand synthesis system (Invitrogen) in a 20 µL reaction volume as described by the supplier.

4.3.3 Bacterial strains, media, growth conditions, and transformations

Escherichia coli DH5α and *Agrobacterium tumefaciens* EHA105 cultures were grown in LB media (1.2% (w/v) tryptone, 1.2% (w/v) NaCl and 0.6% (w/v) yeast extract). Bacterial transformations were performed using the heat-shock method as described in Sambrook et al (1989). Transformants were selected using the appropriate antibiotic as selection on LB plates. Putative positive colonies were cultured and their plasmids isolated and verified by restriction digest. *E. coli* cultures were grown at 37ºC and *A. tumefaciens* cultures at 30°C.

4.3.4 Plasmids, cloning and bacterial transformations

The plasmids pAC-85b, pAC-LYC and pAC-BETA used to perform the functional complementation assays were obtained from F. X. Cunningham (Department of Cell Biology and Molecular Genetics, University of Maryland, MD, USA) and are described in Cunningham et al (1993, 1994) and Cunningham (2002).

The primer pair VvCCD1_5' and VvCCD1_3' was designed according to TIGR sequence TC44975 and used to amplify the *VvCCD1* gene from *Vitis vinifera* L. cv. Pinotage cDNA (Table 2). The resultant PCR amplicon was cloned into the pGEM-T Easy vector system according to the specifications of the supplier (Promega), to generate the plasmid pGEMt-VvCCD1.

Both an overexpression vector and a silencing vector were constructed using the binary pART27 vector as a backbone containing the cauliflower mosaic virus (CaMV) promoter and the octopine synthase gene (*ocs*) terminator (Gleave 1992). The 1715 bp VvCCD1 coding region was excised from pGEMt-VvCCD1 as a *Sal*I/*Spe*I fragment and cloned into the compatible *Xho*I/*Xba*I sites of pART27 to yield pART27-VvCCD1.

The silencing vector was created using the pHANNIBAL vector (Wesley et al. 2001). The construct allows for the transcription of double-stranded RNA in a plant. A 148 bp fragment was PCR-amplified from the 3' untranslated region (UTR) of *VvCCD1* from *V. vinifera* L. cv Sultana genomic DNA using the primer pair VvCCD1_RNAi_5' and VvCCD1_RNAi_3' (Table 2). The pGEM-T Easy vector system was used to clone the PCR amplicon according to the specifications of the supplier (Promega), creating the pGEMt-CCD(RNAi) plasmid. pHANNIBAL was subsequently linearised with *Xho*I and *Eco*RI and ligated with the 136 bp fragment isolated from pGEMt-CCD(RNAi) by *Xho*I and *Eco*RI restriction digests. The resultant plasmid was subsequently digested with *Bam*HI and *Xba*I and the 148 bp *Bam*HI and *Xba*I fragment from pGEMt-CCD(RNAi) was ligated into the corresponding sites. The resultant plasmid, pHANNIBAL-CCD(RNAi), contained a 148 bp inverted repeat of the 3'-UTR of *VvCCD1*. The expression cassette was excised from pHANNIBAL-CCD(RNAi) with *Not*I, and ligated into the corresponding *Not*I site of pART27 yielding the final *VvCCD1* silencing vector, pART27-CCD(RNAi).

4.3.5 Bacterial functional complementation

E. coli DH5α cells were co-transformed with pART27-VvCCD1 and pAC-85b, pAC-LYC or pAC-BETA. The pART27 cloning vector was used as a negative control. The bacterial cultures were inoculated to approximately identical OD_{600} in 5 mL LB media and grown overnight. The culture OD_{600} was recorded and 4.5 mL removed and added to a 20 mL solid phase microextraction (SPME) vial containing 5.5 mL dH_20 and 5 g NaCl, creating a final concentration of 20% (w/v) NaCl. 10 µL of a 3-octanol solution (16 $ng.\mu L^{-1}$) was added as an internal standard (IS) and the vial was sealed with a PTFE/silicon septum. The level of norisoprenoid production by the cultures was measured using head space (HS) SPME GC/MS.

4.3.6 Grapevine transformations, growth conditions and plant material

Grapevine transformation and regeneration was performed according to Franks et al. (1998). Somatic embryonic callus from *V. vinifera* L. cv. Sultana was incubated with *A. tumefaciens* containing either the overexpression or silencing vector. Selection (100 $\mu g.mL^{-1}$ kanamycin) was maintained until *in vitro* rooted plantlets were obtained. Regenerated plantlets were maintained and vegetative propagated on MS media. Plants were then acclimatised in a greenhouse and grown in a commercial soil mixture supplemented with Nitrosol® every 3 weeks. Analyses of gene expression, pigment concentrations and volatile composition were performed on fully expanded leaf tissue. Leaves were flash frozen in liquid nitrogen immediately upon harvesting and stored in the dark at -80°C.

4.3.7 Southern blot analysis

Southern blot analysis was performed using 10-20 µg of genomic DNA extracted from grapevine leaves. The DNA was digested with *Spe*I, separated in a 0.8% (w/v) TBE agarose gel and transferred to a positively charged Hybond-N nylon membrane as described by the supplier (Amersham-Pharmacia Biotech, Buckinghamshire, UK). Probe labelling, hybridisation, and Biotin detection were performed using the Biotin non-radioactive nucleic acid labelling and detection system according to the specifications of the supplier (Roche Diagnostics, Mannheim, Germany).

Table 2. Primers used in this study.

Primer name	Sequence	Tm (°C)	Amplicon size	Use
VvCCD1_5'	GTCGACAAGAAGATGGCGGAGAAGGAGG	64	1715 bp	Amplify the *VvCCD1* coding region from *V. vinifera* L. cv. Pinotage cDNA
VvCCD1_3'	CTACATGCATACCTTACATGGCGG	57		
CCD1_RNAi.5'	TCTAGACTCGAGGAAATCATAGACCGCCATGT	63	148 bp	Amplify a region of the *VvCDD1* 3'UTR from *V. vinifera* L. cv. Sultana genomic DNA
CCD1_RNAi.3'	GGATCCGAATTCAATCCTTTCATAAGTAGCCATAAT	61		
RT-CCD.F	CCATACGGTTTTCATGCCTTC	60	97 bp	Amplify endogenous and transgenic VvCCD1 transcripts during Real-time PCR
RT-CCD.R	TGCATACCTTACATGGCGGTC	60		
RT-GAPDH.F	TGTGATCGACTTGATTGTCCACA	60	114 bp	Amplify a 3'UTR of *VvGAPDH* for normalisation during Real-time PCR
RT-GAPDH.R	GGGACTCTTAACCATATGCCCAA	60		
RT-P27_CCD.F	GAGAGGACACGCTCGACAAGA	60	86 bp	Amplify transgenic *VvCCD1* transcripts during Real-time PCR
RT-P27_CCD.R	CTTACTCGGCTTCGGATCCA	60		

4.3.8 Real-time PCR

Expression analysis of *VvCCD1* was performed via Real-time PCR. Reaction mixtures contained 2 µL of a 1:25 dilution of the first-strand cDNA synthesis reaction as template. The grapevine glyceraldehyde-3-phosphate dehydrogenase (*VvGAPDH*) gene was used as a "house-keeping" gene to normalise expression (Reid et al, 2006). Primers were designed to bind to a region from the *VvGAPDH* 3'-UTR, a region present in *VvCCD1* (endogenous and transgenic), and a region specific to only the transgenic *VvCCD1* (Table 2). Real-time PCR was performed using the Applied Biosystems 7500 Real-time PCR System. SYBR FAST qPCR Kit (Kapa Biosystems, Cape Town, South Africa) was used according to the manufacturer's instructions to visualise the formation of dsDNA in the PCR reactions. The programme for the PCR reactions was: 50°C for 2 minutes; 95°C for 10 minutes; and 40 cycles of 15 seconds at 95°C and 60 seconds at 58°C. Data were analysed using the Applied Biosystems SDS software (version 1.4). All PCR reactions consisted of at least three technical replicates. Relative expression of the *VvCCD1* gene was calculated using the equation as described by Pfaffl (2001), where *E* is the PCR efficiency and CP is the cycle number at which the florescence crosses the base line.

$$(E_{\text{target}})^{\Delta CP\text{target (control-sample)}} / (E_{\text{reference}})^{\Delta CP\text{reference (control-sample)}}$$

To calculate the PCR efficiency for each primer pair, real-time PCR was performed on a dilution series of template DNA and the CP values plotted relative to the template concentration. The slope of these graphs was then used to determine the PCR efficiency according to the equation describe by Pfaffl (2001), $E = 10^{(-1/\text{slope})}$.

4.3.9 Leaf pigment extraction

Photosynthetic pigments of leaves were analysed using the method described in Chapter 3. Briefly, ground frozen tissue from leaves (±20 mg) was extracted in 2.0 mL micro-centrifuge tubes in an 8°C laboratory taking care to minimise exposure to air and light. The leaf samples were suspended in 1.8 mL acetone and the internal standard, β-apo-caroten-8-al, was added to all extracts (2 µg). The tubes were vortexed for 30 minutes, followed by pelleting of the tissue debris via centrifugation (10,000 g, 3 minutes). A 300 uL aliquot of the acetone extract was removed and added to 1 mL of extraction buffer and washed by vortexing for 5 minutes. Ethyl acetate (200 µL) was added, vortexed briefly followed by centrifugation (10,000 g, 5 minutes) to partition the mixture. A 50 µL aliquot of the 150-200 µL upper ethyl acetate

phase (containing pigment) was removed, added to 200 µL methanol containing 0.125% (w/v) BHT, mixed by vortexing and 200 µL subsequently transferred to amber HPLC vials and sealed.

4.3.10 HPLC conditions

The chromatographic conditions described in Chapter 3 were used to separate the major pigments extracted from grapevine tissue. All pigments were separated by RP-HPLC on an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a DAD system. A YMC30 column (250 mm × 2.1 mm) and YMC30 guard cartridge (10 mm × 2.1 mm, particle size 3 µm) from YMC Europe (Schermbeck, Germany) were used. Chemstation software for LC3D (Rev.A.10.01[1635]; Hewlett-Packard, Waldbronn, Germany) was used for data processing.

A binary mobile phase of 3% (v/v) ddH_2O in methanol containing 0.2% (w/v) ammonium acetate and 0.05% (v/v) triethylamine (solvent A) and TBME containing 0.05% (v/v) triethylamine (solvent B) was employed. The flow rate was 1 $mL.min^{-1}$ with the temperature maintained at 25°C. The elution program was isocratic at 20% B for 12 minutes followed by a linear increase from 20% to 50% B in 6 minutes, isocratic at 50% B for 4 minutes, a linear increase to 68% B in 2 minutes, isocratic at 68% for 4 minutes followed by a linear decrease to 20% B in 2 minutes. The column was equilibrated for 15 minutes at the starting condition before each injection.

4.3.11 Extraction of volatile leaf apocarotenoids

Volatiles were extracted according to the method described by Lücker et al. (2001) with modifications. Frozen, ground leaf tissue (200 mg) was placed in a 20 mL SPME vial and 10 mL of 20% (w/v) NaCl containing 160 ng 3-octanol (IS). The vial was sealed with a PTFE/silicon septum.

4.3.12 HS-SPME GC/MS conditions

Samples in sealed SPME vials were heated to 80°C and incubated for 5 minutes before the injection needle was pierced through the septum exposing the divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 µm coated solid phase microextraction (SPME) fibre (Supelco, Belfonte, PA, USA) to the headspace of the sample. During extraction the sample was stirred at 500 rpm and maintained at 80°C. After

15 minutes the fibre was removed and injected into the GC inlet where it was desorbed for 10 minutes at 260°C.

Extracts were analysed using an Agilent 6890 Gas Chromatograph coupled to a Waters GCT Time of Flight (TOF) Mass Spectrometer (MS) (Waters Corporation, Milford, MA, USA) Waters Masslynx GC/MS workstation software were used to analyse the data. The injector port was heated to 260°C and splitless injection (with a purge time of 3 minutes) was used. Separation was performed on an HP5MS (Agilent Technologies, Palo Alto, CA) column (30 m L x 0.25 mm i.d. x 0.25 μm f.t.) with helium as the carrier gas at a constant flow of 1 $mL.min^{-1}$. The initial oven temperature was 40°C for 5 minutes, after which the temperature was increased by 5°$C.min^{-1}$ to 150°C and then at 10°$C.min^{-1}$ to 280°C (held for 2 minutes). Ionisation was in electron impact mode with an electron energy of 70eV. The MS detector was set as follows: The transfer line, ion source and trap temperatures were 250, 180 and 150°C, respectively. The mass range was 35 to 650 m/z, with a scan rate of 4 $scans.s^{-1}$. Compound identification was performed by both comparisons to the retention times of authentic standards and to the NIST05 mass spectral library (National Institute of Standards, USA).

4.4 Results

4.4.1 Bacterial functional complementation assay

Carotenoid accumulating *E. coli* strains were co-transformed with pART27-VvCCD1. Enzyme activity was monitored by measuring the formation of the various norisoprenoids via HS-SPME GC/MS. The phytoene-accumulating strain (transformed with pAC-85b) showed no significant norisoprenoid production when compared to the control strains (data not shown), while strains accumulating lycopene and β-carotene (transformed with pAC-LYC and pAC-BETA, respectively) showed a significant increase in norisoprenoid production when co-transformed with pART27-VvCCD1 (Figure 1a). The lycopene-accumulating strains showed a 6-fold increase in MHO and a 20-fold increase in pseudoionone production in strains co-transformed with pART27-VvCCD1 (relative to the control strains). A 15-fold increase in β-ionone formation relative to the control strains was observed in strains accumulating β-carotene co-transformed with pART27-VvCCD1.

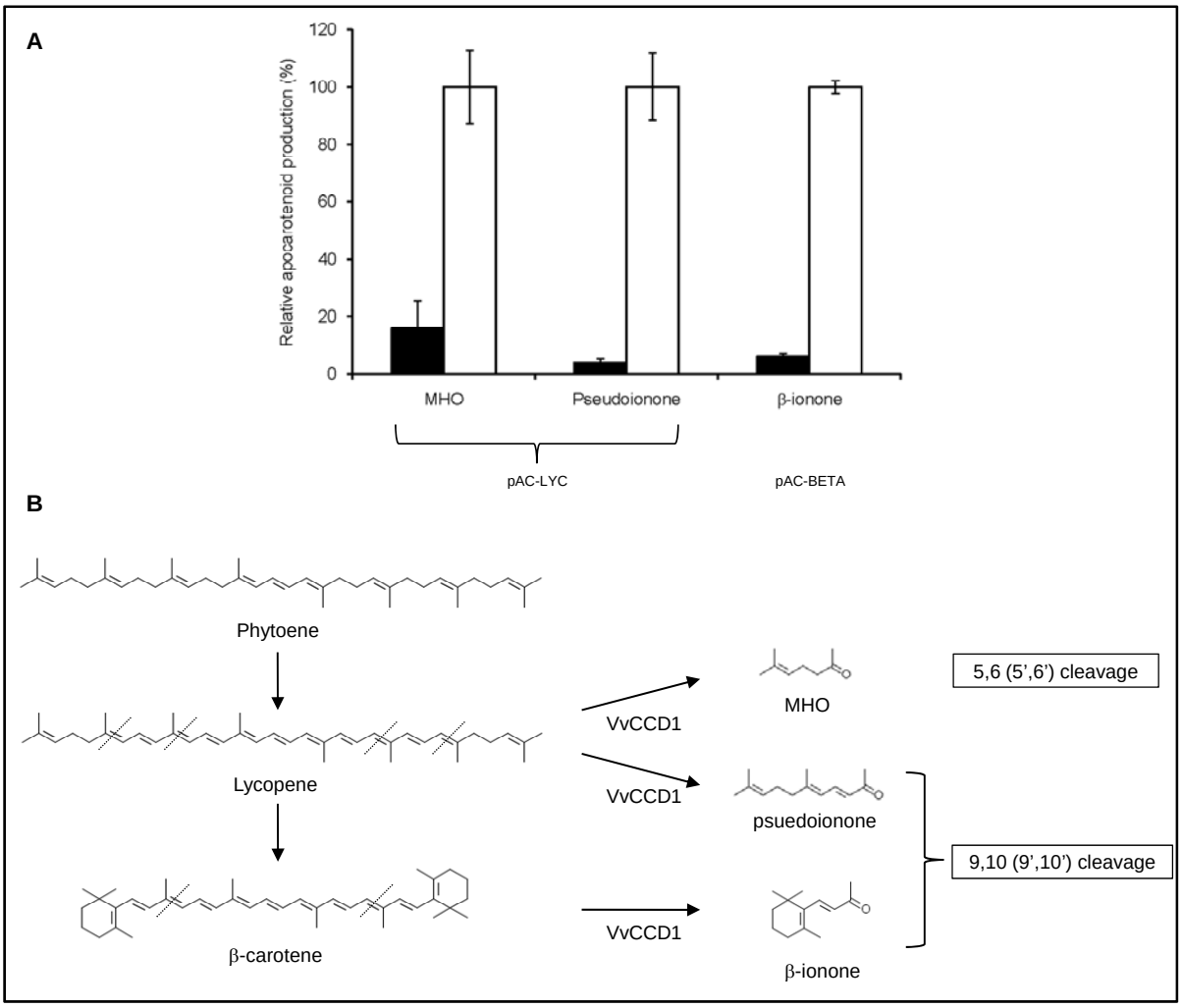

Figure 1. *E. coli* functional complementation of VvCCD1. (A) Strains accumulating either lycopene (transformed with pAC-LYC) or β-carotene (transformed with pAC-BETA) were co-transformed with either pART27-VvCCD1 (□) or the control plasmid, pART27 (■) and the relative production of various norisoprenoids measured via HS-SPME GC/MS (n = 3). (B) Results from transformation of carotenoid accumulating *E. coli* strains indicates VvCCD1 is able to catalyse the cleavage of lycopene at the 5,6 (5',6') and 9,10 (9',10') double bonds forming 6-methyl-5-hepten-2-one (MHO) and psuedoione, respectively, and β-carotene at the 9,10 (9',10') double bonds, forming β-ionone.

4.4.2 Genetic analysis of transformed plants

Transformation of *V. vinifera* L. Sultana with pART27-VvCCD1 was confirmed with a Southern hybridisation (Figure 2). Digestion of the gDNA with *Spe*I resulted in a single hybridisation band per gene copy as the restriction enzyme cut outside of the area of hybridisation. The blot shows two hybridisation events for the wild type line, indicating that Sultana contains two copies of *VvCCD1*. Analysis of the transgenic plants showed that six of

the nine lines are independent, with a transgene copy number ranging from one to four. Lines CCD1-10 and CCD1-12 are clonal, as are lines CCD1-15, CCD1-17, and CCD1-19.

Confirmation of the transformation of the pART27-CCD(RNAi) construct into the Sultana genome was confirmed by PCR (data not shown) with primer binding sites situated in the CaMVp region and the *VvCCD1* 3'-UTR of the transgenic cassette. Twelve lines were shown to be positive for transformation with the silencing cassette.

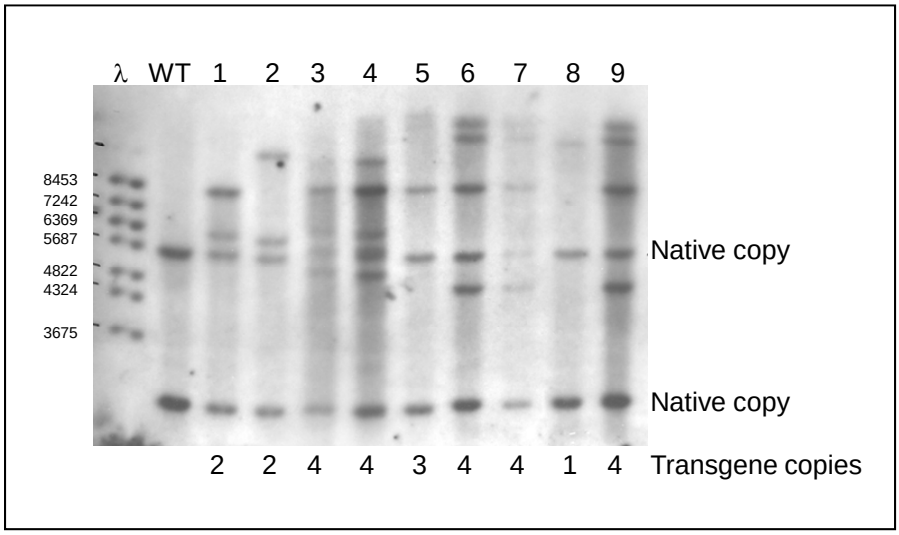

Figure 2. Southern hybridisation blot confirming integration of pART27-VvCCD1. Band sizes (in bp) of λ DNA digested with *Bst*E II (λ) is shown. gDNA was digested with *Spe*I. Two hybridisation events in wild-type (WT) indicate two copies of *VvCCD1* in Sultana. Lane 1 is loaded with gDNA from line CCD1-01; lane 2: CCD1-02; lane 3 CCD1-10; lane 4: CCD1-12; lane 5: CCD1-14; lane 6: CCD1-15; lane 7: CCD1-17; lane 8: CCD1-18; lane 9: CCD1-19. Estimated transgene copy numbers are displayed.

4.4.3 Expression analysis

The level of *VvCCD1* expression in the transgenic lines was monitored via RT-PCR. Of the six lines independently transformed with pART27-VvCCD1, only two lines showed significant overexpression of *VvCCD1* (up to an 85% increase relative to the expression seen in wild-type) (Figure 3a). Real-time expression analysis demonstrated transcription of the endogenous gene as well as the introduced transgene in all the lines, yet four lines displayed total *VvCCD1* gene expression levels which were not significantly increased (when compared to the wild-type) (Figure 3a).

Of the twelve lines positively transformed with pART27-CCD(RNAi), seven showed significant silencing when compared to the wild-type lines (Figure 3b). Silencing of *VvCCD1*

of up to 85% (relative to wild-type expression) was observed. Further metabolite analyses were performed on the plant lines with expression levels that differed significantly from the wild-type.

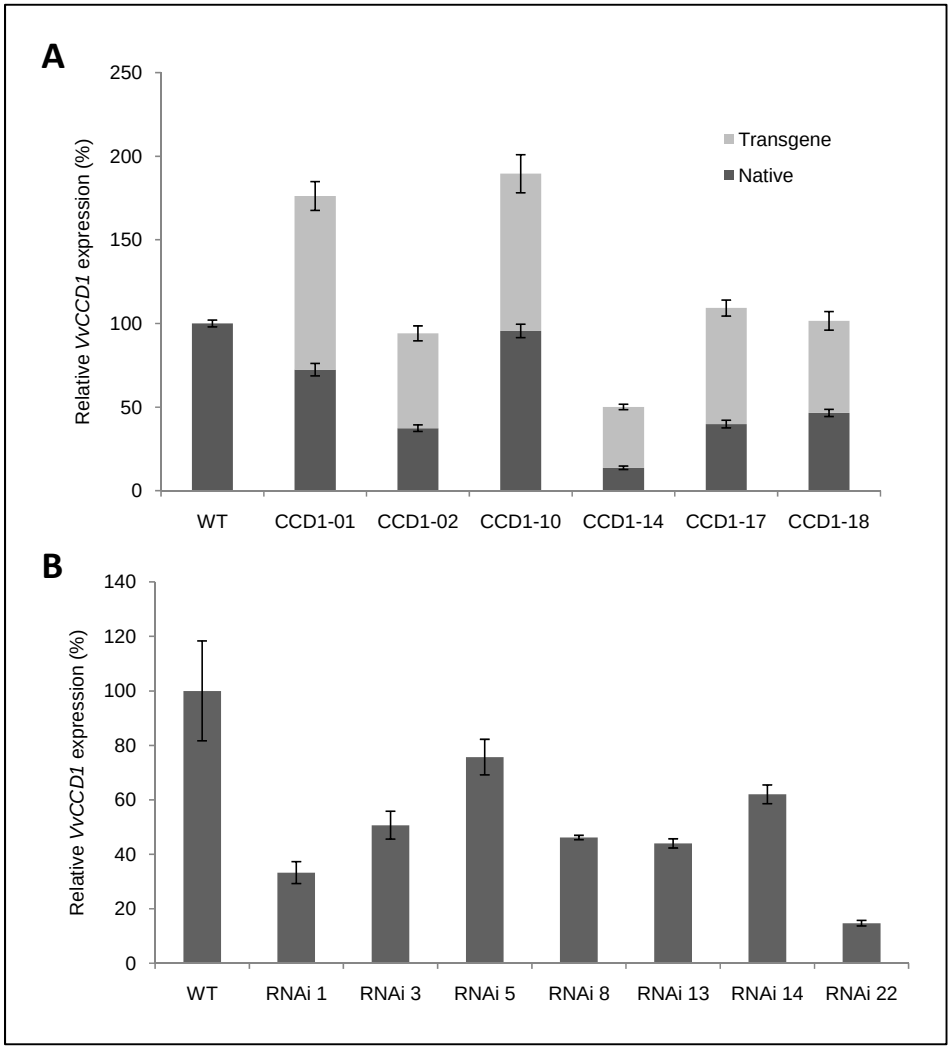

Figure 3. Real-time PCR analysis of *VvCCD1* expression in the transgenic grapevine population. (A) Expression of endogenous (■) and transgenic (■) *VvCCD1* in lines transformed with the overexpression cassette (CCD1) (n = 3). (B) Expression of *VvCCD1* in lines transformed with the silencing cassette (RNAi) (n = 3). Data are expressed relative to wild-tpye (WT) expression and normalised to *VvGAPDH* expression.

4.4.4 Pigment and norisoprenoid analysis

Pigments were extracted from grapevine leaf tissue and analysed via RP-HPLC. Figure 4 shows a strong linear correlation (r^2 = 0.9017) between total chlorophylls and total carotenoids present in the leaves of the wild-type and transgenically modified lines. The photosynthetic pigment concentrations in the transgenics (both silenced and overexpressed

lines) were compared to *VvCCD1* expression (Figure 5). No correlation between gene expression and the concentration of pigments in the leaves was observed. Norisoprenoids extracted from the leaf tissue of the grapevine lines were expressed relative to the recovery of the IS and compared with *VvCCD1* expression (Figure 6). As in the case of carotenoids no correlation was observed between norisoprenoid levels and *VvCCD1* expression. This lack of correlation was confirmed with ANOVA analysis performed using Statistica 8 software (Statsoft, Tulsa, UK). However, when levels of MHO were compared to geranylacetone levels a strong correlation was observed (Figure 7a). A similar correlation was also seen between α-ionone and β-ionone levels (Figure 7b). β-ionone levels, however, showed significantly weaker correlation to MHO (Figure 7c).

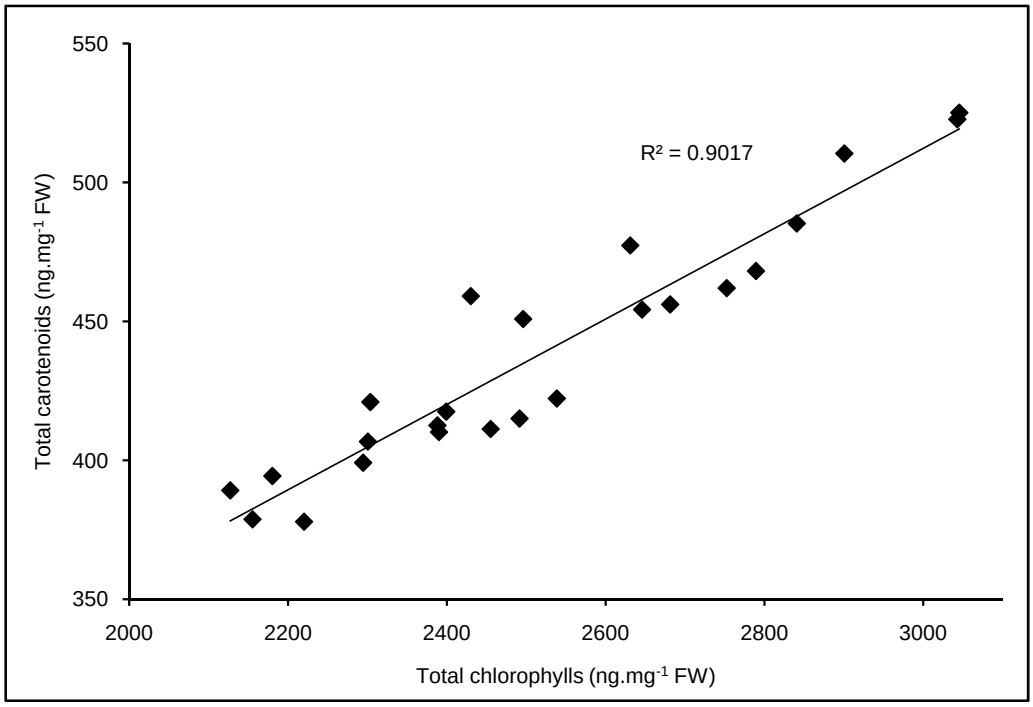

Figure 4. Relationship of chlorophylls and carotenoids in the grapevine population. HPLC analysis of the transgenic and wild-type grapevine used in the study showed a strong correlation between the total carotenoid concentration and the total chlorophyll concentration found in the leaves.

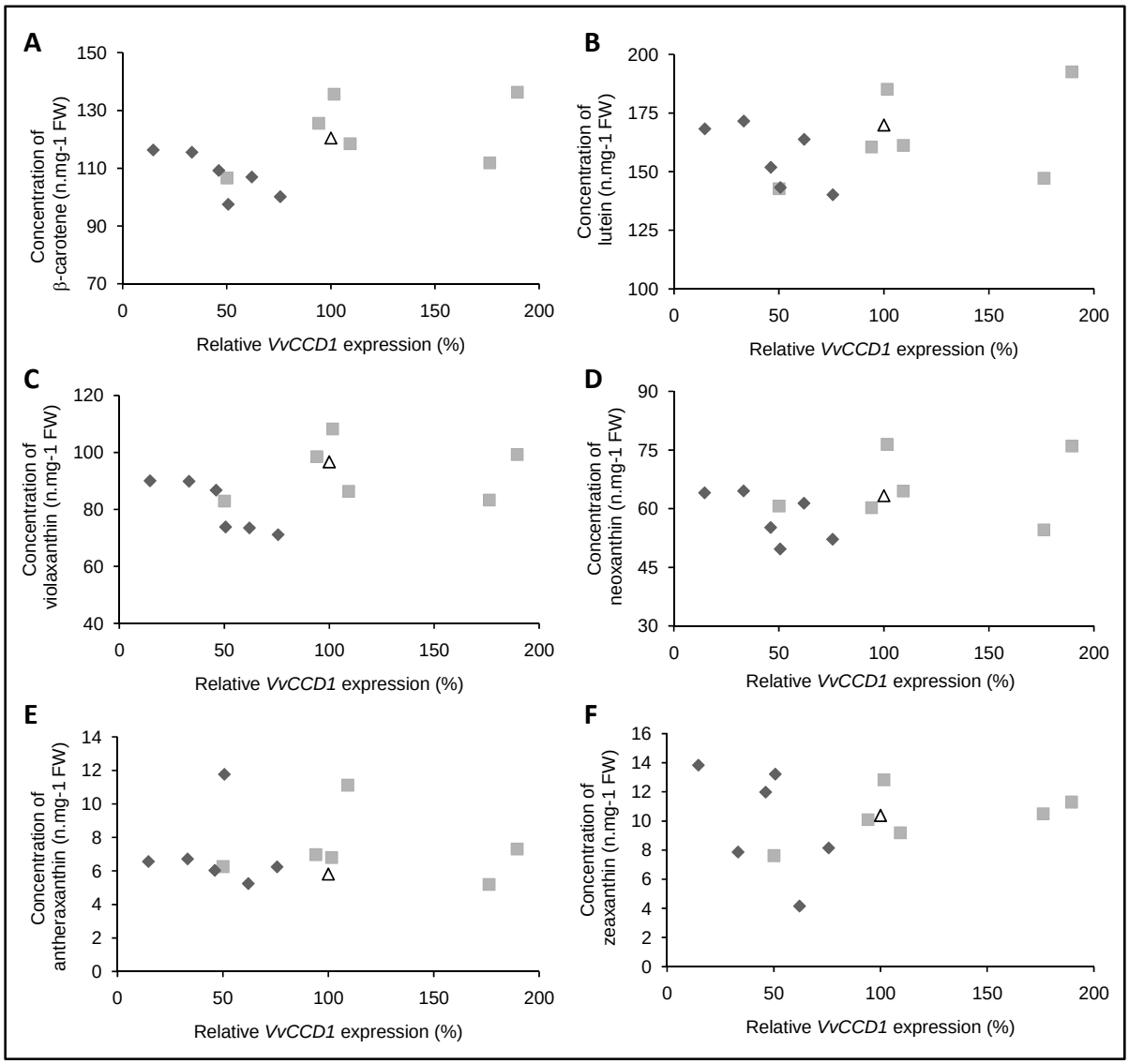

Figure 5. Relationship between carotenoid concentration and *VvCCD1* expression in grapevine leaves. None of the major carotenoids in the leaves of the grapevine population measured via HPLC showed a correlation with *VvCCD1* expression. The concentration of β-carotene (A), lutein (B), violaxanthin (C), neoxanthin (D), antheraxanthin (E) and zeaxanthin (F) found in wild-type (△), silenced (◆) and overexpression (■) lines is shown.

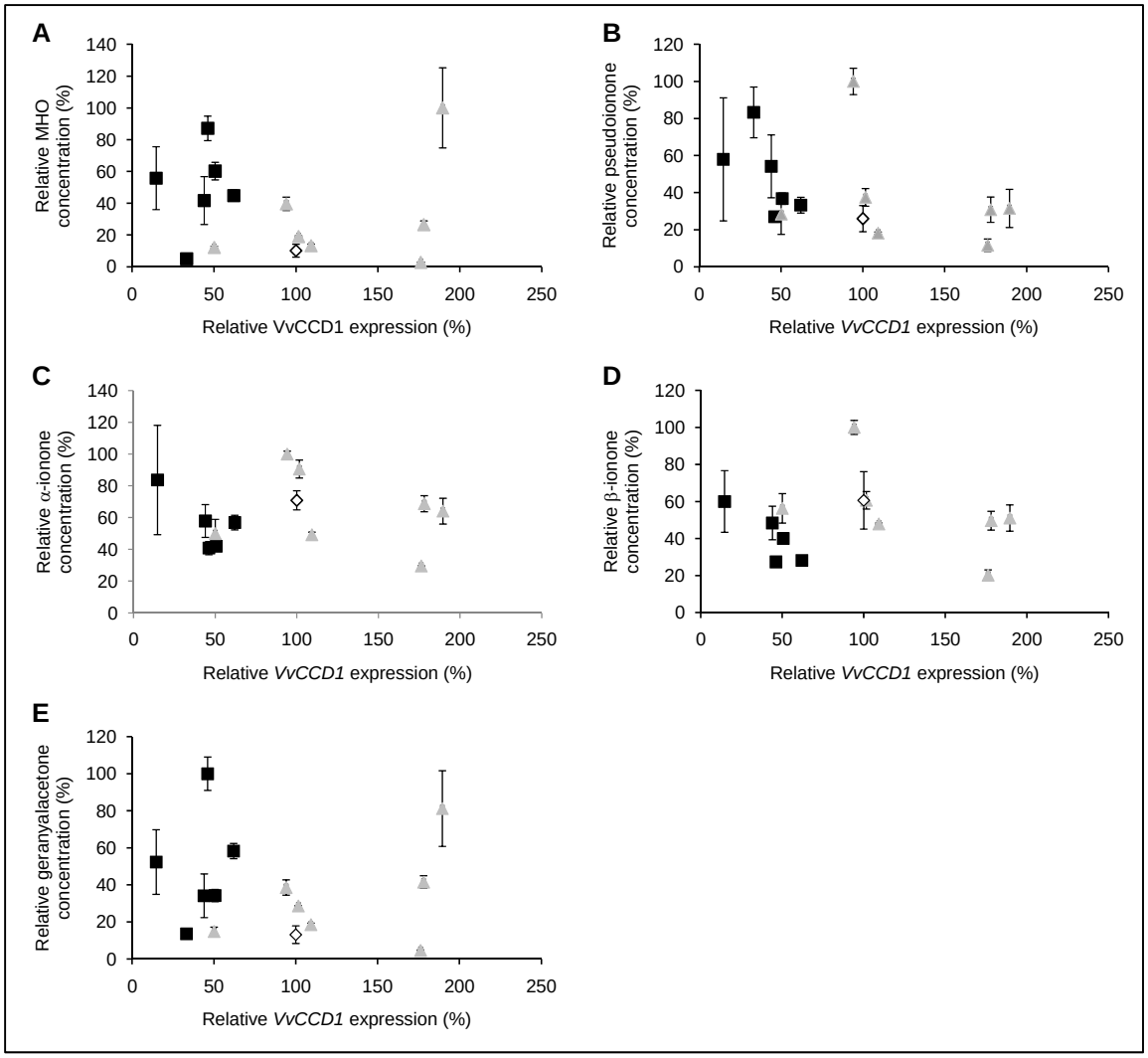

Figure 6. Relationship between apocarotenoid concentration and *VvCCD1* expression in grapevine leaves. None of the apocarotenoids in the leaves of the grapevine population measured via GC/MS showed a correlation with *VvCCD1* expression. The concentration of 6-methyl-5-hepten-2-one (MHO) (A), pseudoionone (B), a-ionone (C), b-ionone (D), and geranylacetone (E) found in wild-type (◇), silenced (■) and overexpression (▲) lines is shown.

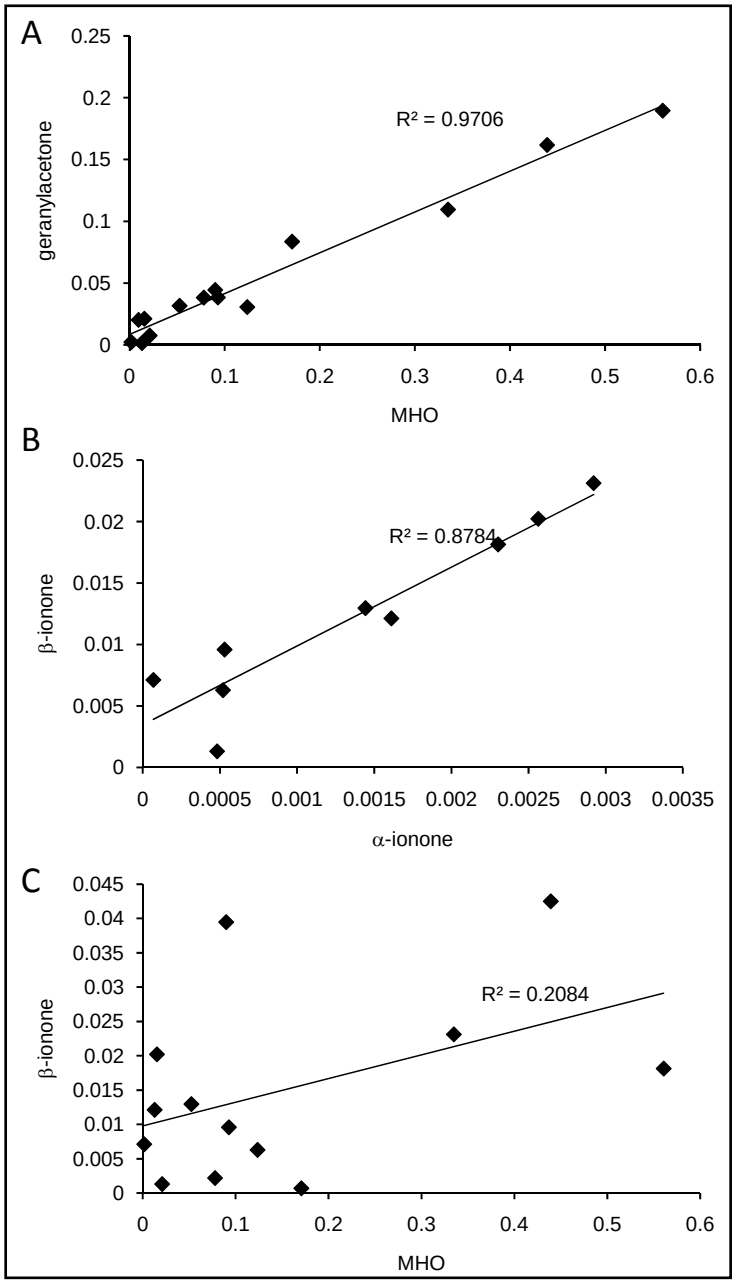

Figure 7. Correlation between pools of apocarotenoids in grapevine leaves. GC/MS analysis of leaf tissue from the grapevine population showed a strong correlation between geranylacetone and 6-methyl-5-hepten-2-one (MHO) levels (A) and between β-ionone and α-ionone levels (B), but not between β-ionone and MHO levels (C). Data are expressed relative to the IS.

4.5 Discussion

4.5.1 Chromosomal organisation of VvCCD1

There are currently two draft grapevine genome sequences, both from Pinot noir: PN40024, an inbred near homozygous (93%) clone (Jaillon et al. 2007); and ENTAV115 a highly heterozygous clone (Velasco et al. 2007). Southern blot analysis of native *VvCCD1* confirmed Sultana possesses two copies of the gene (Figure 2), in agreement with the PN40024 genomic sequence that shows a tandem duplication of *VvCCD1* on chromosome 13, with the two copies situated approxiamatly 78 kbps from each other. The two *VvCCD1* genomic copies have a 96% identity and appear to be a tandem duplication event.

4.5.2 Protein analysis

In silico protein localisation software predicts a cytosolic localisation for VvCCD1. Orthologues for VvCCD1 have been predicted to be localised to the cytosol. Protein alignments between CCD1 orthologues display a highly conserved amino acid sequence (Table 1). Four crucial histidine residues that are conserved in CCD orthologues were identified in *VvCCD1* (Kloer & Schulz 2006). These amino acids are involved in the binding of an iron cation (Fe^{2+}) which has been shown to be a co-factor for the carotenoid cleavage reaction (Poliakov et al. 2005).

4.5.3 VvCCD1 enzyme activity

Mathieu et al. (2005) have previously shown, in *in vitro* studies, that VvCCD1 cleaves zeaxanthin and lutein, forming the norisprenoid 3-hydroxy-β-ionone, while CCD1 homologues in other plant species have been shown to cleave a broad variety of carotenoids (Table 1). The production of MHO, pseudoionone and β-ionone in carotenoid accumulating *E. coli* strains co-transformed with pART27-VvCCD1 further illustrates the promiscuity of the enzyme (Figure 1). Lycopene and β-carotene can now be added to the list of substrates for VvCCD1 (Table 1). Interestingly lycopene can be cleaved at both the 9,10 (9',10') double bond site and the 5,6 (5',6') double bond site, forming pseudoionone and MHO, respectively (Figure 1). It would seem that 9,10 cleavage is more likely in *E. coli* as the production of pseudoionone in the strains was significantly greater than MHO production (Figure 1a). Maize, *Arabidopsis*, and tomato CCD1 enzymes all show this 5,6 cleavage of lycopene (Vogel et al. 2008, Simkin et al. 2004). Cleavage of phytoene at the 5,6 (5',6') bond would also result in MHO formation, but this cleavage was not observed in phytoene-accumulating strains co-transformed with pART27-VvCCD1 (data not shown).

A possible explanation for substrate recognition lies in the degree of saturation of the carotenoids (Vogel et al. 2008). While phytoene possesses both the 5,6 (5',6') and 9,10 (9',10') double bond sites that are cleaved in the carotenoids that are CCD1 substrates, it lacks double bonds at the 7,8 (7',8') and 11,12 (11',12') positions (Figure 8). The desaturation of phytoene to lycopene, and the subsequent introduction of these double bonds allows for cleavage of the carotenoid. Additionally it has been suggested by Vogel et al. (2008) that CCD1 cleavage only occurs when methyl groups flank the double bond being cleaved. A methyl group must be found immediately adjacent to the double bond and four carbon atoms central to the site of cleavage. In the case of 9,10 cleavage a methyl group must be situated on the C-9 and C-13 atom (Figure 8).

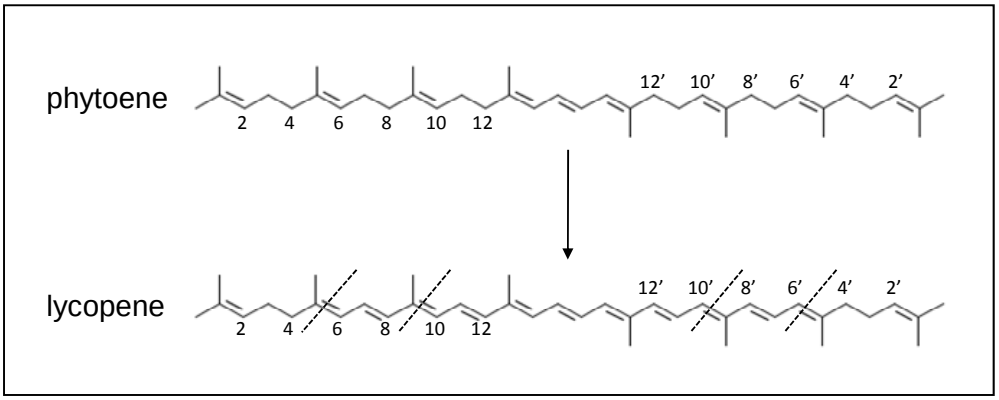

Figure 8. Schematic illustrating cleavage sites of VvCCD1. Despite phytoene possessing double bonds at the 5,6 (5',6') and 9,10 (9',10') positions, no cleavage by VvCCD1 is observed. Enzymatic de-saturation of phytoene to lycopene results in the formation of additional double bonds at the 7,8 (7',8') and 11,12 (11',12') positions. These adjacent double bonds appear to be necessary for VvCCD1-mediated cleavage.

4.5.4 Altering VvCCD1 transcript levels

A transgenic grapevine population with altered levels of *VvCCD1* expression was generated to allow for the *in vivo* characterisation of the role *VvCCD1* plays in grapevine. In the case of overexpression of the gene, despite being able to generate six independent lines stably transformed with the overexpression cassette (pART27-VvCCD1) (Figure 2), only two of these lines (CCD1-01 and CCD1-10) showed a significant increase in total *VvCCD1* expression (85%) (Figure 3a). Twelve grapevine lines were transformed with the silencing construct. Of these lines seven showed varying degrees of *VvCCD1* silencing. The most silenced line (RNAi 22) had a reduction in *VvCCD1* mRNA of 85% while the lowest level of

silencing observed was a 25% reduction in *VvCCD1* expression (relative to the wild-type). The remaining lines showed varying degrees of silencing between these two values.

The silencing construct drives transcription of an inverted repeat cloned from the 3'-UTR of VvCCD1. This RNA fragment then folds on itself creating a dsRNA fragment that exploits the plants' natural defence mechanism by inducing RNAi silencing of transcripts homologous to the inverted repeat reviewed in Wesley et al. (2001). At the time of design of the RNAi construct the sequenced grapevine genome was not available. To limit the silencing effect exclusively to *VvCCD1*, and avoid silencing of the large family of *CCD*s (as found in *Arabidopsis*), the fragment chosen to initiate silencing was designed in the less conserved 3'-UTR of *VvCCD1*. Wesley et al. (2001) show that RNAi-mediated silencing is equally efficient when using constructs targeting the coding region, 5'-UTR or 3'-UTR. The resultant population, consisting of lines exhibiting both overexpression and silencing of *VvCCD1*, will serve as a useful genetic resource for the *in planta* study of *VvCCD1* and its biological roles.

4.5.5 Pigment and apocarotenoid analysis of grapevine lines

RP-HPLC analysis of pigments extracted from the transgenic population showed no correlation between *VvCCD1* expression levels and the concentration of carotenoids in grapevine leaf tissue under the conditions tested (Figure 5). Similarly no correlation between gene expression and leaf norisoprenoid levels were observed in grapevine leaves (Figure 6). Total carotenoid content however, correlated very strongly with the total chlorophyll content of the leaves (Figure 4). This is expected as carotenoids are primarily involved in photosynthesis specifically in vegetative tissue (Cunningham 2002). An increase in chlorophyll concentration is indicative of increased photosynthetic activity (Lichtenthaler et al. 1981), which in turn puts more demand on the carotenoids involved in light-harvesting and photoprotection. It is therefore not surprising that the carotenoid pathway is tightly regulated in leaf tissue, as loss of these crucial pigments would lead to a non-viable plant.

The positive correlation observed between MHO and geranylacetone (Figure 7a) and between α-ionone and β-ionone (Figure 7b), but not between the two groups (Figure 7c) suggests that these groups of apocarotenoids are derived from different sources. This would suggest that some type of segregation between these two pools of substrates exists, where each pool may be differentially exposed to an agent that is able to cleave carotenoids. In this case MHO and

geranylacetone are both derived from non-cyclic carotenoids, whereas α-ionone and β-ionone are derived from downstream carotenoids that have undergone varying degrees of cyclisation.

4.5.6 Mechanism of control

The protection from manipulation of the carotenoid pathway, specifically from altered levels of *VvCCD1* transcript, can occur through a number of different mechanisms. Significant overexpression of the *VvCCD1* was only observed in 30% of the Southern positive grapevine lines. This was not due to a non-functional construct, as RT-PCR experiments that discriminated between the endogenous and the transgenic *VvCCD1* illustrated (Figure 3a). Additionally the same construct was able to drive expression of *VvCCD1* in *E. coli* resulting in the translation of a functional protein (Figure 1). There however appeared to be a form of post-transcriptional gene silencing (PTGS) occurring in the transgenic plants. This is not uncommon in transgenic plants with excessively high numbers of transgene intergrations, although the mechanism of silencing is not well understood. The population consists of lines ranging from a single transgene insert to four inserts. There was, however, no correlation between the number of transgene inserts and the level of silencing observed. One possible mechanism of this silencing could be PTGS, a defence mechanism in plants, whereby the plant has the ability to recognise and destroy dsRNA. This is a crucial defence mechanism against virus attack. Dalmay et al. (2000) have proposed a gene in *Arabidopsis* (*SDE1*) involved in the PTGS of transgenes rather than viral-encoded genes. *AtSDE1* codes for an RNA polymerase which is able to synthesise dsRNA and thus initiate PTGS from single-stranded RNA-derived from a transgene. A *V. vinifera* homologue may be responsible for a similar function.

In spite of the partial silencing of most of the overexpression lines, lines were generated that exhibited overexpression of VvCCD1. These lines together with the lines that showed successful silencing contributed to a population of plants that exhibited a wide range of *VvCCD1* expression. There was a 12-fold difference in gene expression from the most silenced line to the most overexpressed line. Despite this range in *VvCCD1* expression no direct phenotype based on known VvCCD1 enzyme action was observed in vegetative tissue. Mathieu et al. (2005) monitored ripening berries and found a similar discrepancy between *VvCCD1* expression and apocarotenoid concentrations. In their studies they observed that an increase in apocarotenoids occurred a week after the increase in *VvCCD1* transcripts.

No VvCCD1 protein quantification was performed on the plant lines in this study, so it is not possible to quantify the transcription vs translation of *VvCCD1*. There are studies, however, that show a positive correlation between mRNA levels and protein levels is far less common than often assumed (Roberts 2002). Additionally, conditions inhibiting or activating enzyme activity independent of protein concentration may play a role. This has been observed in the well characterised xanthophyll cycle involving the conversion of zeaxanthin to violaxanthin catalysed by zeaxanthin epoxidase (ZEP), and the conversion of violaxanthin to zeaxanthin catalysed by violaxanthin de-epoxidase (VDE) (Demmig-Adams & Adams 1996). ZEP is active at neutral pH which occurs during low light conditions while VDE is active under low pH conditions casued by excess light. Enzyme activity in this case is therefore independent of mRNA levels or protein concentration, but rather relies on favourable conditions for enzyme activity.

A more likely and important factor that could prevent an expected phenotype is the compartmentalisation of the plant cell. *In silico* analysis suggest that VvCCD1 is localised in the cytosol. This has been shown to be the case for many of its homologues (Vogel et al. 2008). The carotenoid substrates CCD1 acts upon, however, are situated in the chloroplast membrane (Cunningham 2002), and are thus inaccessible to the enzyme. A chloroplast targeted transformation approach may be able to overcome this problem, but would likely lead to a plant with severely diminished photosynthetic ability.

Previous attempts to manipulate the levels of *CCD1* transcripts have also led to phenotypes inconsistent with the observed *in vitro* activity of CCD1 (Floβ et al. 2008, Simkin et al. 2004). Simkin et al. (2004) reduced *LeCCD1* mRNA levels by up to 90% in tomato leaves and fruit using an antisense construct, but did not observe any change in the carotenoid concentration in fruit with reduced *CCD1* levels, and only a 50% decrease of β-ionone in selected silenced lines. No data was presented for carotenoid and norisoprenoid levels in tomato leaves with reduced *CCD1* levels. This observation, as well as the similar results obtained in this study, suggest that the *in planta* role of *CCD1* may not be maintenance of carotenoid levels. A similar reduction of the norisoprenoids observed in tomato fruit with reduced *CCD1* transcripts was not seen in grapevine leaves (Figure 6). This is not surprising when the organs being investigated are compared. The leaf, being the primary photosynthetic organ of the plant, needs to maintain this function, while the tomato fruit is a specialised reproductive organ. The correlation observed between chlorophyll concentration and

carotenoid concentration in the leaf (Figure 4) indicates that the level of photosynthetic activity of the tissue has a much greater influence on the concentration of carotenoids. In the chromoplasts of fruit the need for photoprotection of the chlorophylls is absent and the segregation of CCD1 and its carotenoid substrates is possibly diminished. However, despite the predicted cytosolic localisation of CCD1 and its apparent lack of transcript correlation with carotenoid and norisoprenoid concentrations, norisoprenoids are still formed in the leaves (Figure 5 and 6). This suggests another mechanism of action for the cleavage of carotenoids and the production of norisoprenoids. One possible mechanism may be the free radical-mediated carotenoid cleavage observed by (Wache et al. 2003). They were able to demonstrate 9,10 cleavage of β-carotene *in vitro* leading to the formation of β-ionone as a result of oxidation. Another possible candidate for the mechanism of carotenoid cleavage is suggested by Floß et al. (2008). They were able to reduce *MtCCD1* transcript levels by 90% via an RNAi strategy in the hairy roots of *Medicago truncatula*. This led to an increase in C_{27} apocarotenoids as opposed to C_{40} carotenoids, indicating C_{27} apocarotenoids to be the *in planta* substrate for CCD1 enzymes, while another mechanism is at work contributing to cleavage of C_{40} carotenoids. Floß et al. (2008) suggest an *M. truncatula* orthologue of AtCCD7 is able to cleave C_{40} carotenoids. AtCCD7, an *Arabidopsis* protein, has been shown to cleave both β- and ζ-carotene and is localised to the plastid (REF). As opposed to the symmetrical cleavage observed by CCD1 homologues, CCD7 cleaves asymmetrically at the 9,10 double bond forming a C_{13} norisoprenoid and a C_{27} aldehyde (β-ionone and β-apo-10'-carotenal in the case of β-carotene cleavage). The C_{27} aldehyde may then be transported out of the chloroplast where, in the presence of CCD1, it will be possible to be cleaved further. A homologue for *AtCCD7* exists in *V. vinifera* on chromosome 15 (Genbank accession XP_002274198.1) and displays a 78% identity to *AtCCD7* on the amino acid level.

4.5.7 Conclusions and future prospects

In grapevine leaves VvCCD1 enzyme activity appears to be under various levels of control. The importance of the carotenoid pigments for effective photosynthesis is the most likely reason for this control. The control is applied to both the transcript level, where a form of PTGS is observed, and possibly the protein level, where sub-cellular compartmentalisation may prevent interaction between the enzyme and its carotenoid substrates. Despite the fact that VvCCD1 has the ability to cleave multiple carotenoids in *E. coli*, the *in planta* substrates for VvCCD1 cleavage may be C_{27} apocarotenoids produced through cleavage by enzymatic action (CCD7 is suggested by Floß et al. (2008)) or photo-oxidation, and subsequently

transported from the chloroplast to the cytosol. In either case grape berries would likely display a phenotype more closely correlated to *VvCCD1* expression due to the increased osmotic stress that occurs during ripening resulting in leaky membranes and the concomitant degradation of chloroplasts. Future studies on grape berries from the transgenic lines generated in this study will be of great importance in futher elucidating the *in planta* function of *VvCCD1*. Additional studies should include *in situ* protein hybridisation as well as analysis of tissue with less of a need for the strict maintenance of carotenoid concentrations, such as reproductive organs and etiolated plants.

4.6 Acknowledgements

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4.7 References

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Chapter 5

General discussion

and conclusions

5.1 General discussion and conclusions

Carotenoid cleavage dioxygenases (CCDs) are an important class of catabolic enzymes occurring throughout the natural world. In plants the CCD family catalyses cleavage of carotenoids at specific positions, leading to the formation of apocarotenoids. Carotenoids are important accessory pigments involved in light harvesting during photosynthesis. Biosynthesis of carotenoids occurs in the plastid where they are found bound in complexes with chlorophyll. CCD-derived apocarotenoids play a number of important roles in the plant, and include the phytohormone abscisic acid, and the flavour compound β-ionone.

Typically, CCD1 proteins have been shown to produce flavour and aroma compounds *in vitro*, but details regarding substrate specificities, as well as *in planta* functions are still limited. The objectives of this study were to functionally analyse a CCD1-encoding gene from grapevine (*Vitis vinifera* L.). To this end, both *in vitro* and *in planta* analyses were performed.

In previous studies *VvCCD1* has been demonstrated *in vitro* to cleave zeaxanthin and lutein forming the volatile flavour compound, 3-hydroxy-β-ionone (Mathieu et al. 2005). Transformation of carotenoid-accumulating *Escherichia coli* strains with *VvCCD1* led to further characterisation of the substrate specificity of the enzyme (Chapter 4). While phytoene could not be cleaved by VvCCD1, both lycopene and β-carotene served as substrates. Cleavage of β-carotene at the 9,10 (9',10') position resulted in the formation of β-ionone, while lycopene was cleaved at both the 5,6 (5',6') position forming 6-methyl-5-hepten-2-one and at the 9,10 (9',10') position forming pseudoionone. Similar cleavage of lycopene at the 5,6 (5',6') double bond by CCD1 has been observed in maize, *Arabidopsis*, and tomato (Simkin et al. 2004, Vogel et al. 2008). VvCCD1 (similarly to other characterised CCD1 orthologues) is therefore able to cleave multiple carotenoids substrates *in vitro* that include lycopene, lutein, β-carotene and zeaxanthin, but not phytoene.

In order to characterise the biological role of *VvCCD1*, transgenic *V. vinifera* cv. Sultana lines were generated with altered levels of *VvCCD1* expression. Somatic embryogenic callus from *V. vinifera* was transformed with either a *VvCCD1* overexpression or silencing cassette via *Agrobacterium*-mediated transformation. Putative transformed plant lines were analysed for the presence of the integration cassettes via PCR and/or Southern hybridisation. Leaf tissue from lines showing successful integration were further genetically characterised for

expression of *VvCCD1* via Real-time PCR. The genetically characterised population was analysed for carotenoid concentrations and volatile apocarotenoid formation in the leaves.

Metabolic characterisation of the transgenic lines required the development of a method for pigment extraction and quantification (Chapter 3). While a number of techniques for pigment quantification via HPLC exist, the diversity between tissues from different organisms necessitated the development of an improved method. Care was taken to identify and avoid conditions leading to degradation of the sensitive photosynthetic pigments during the extraction process. Light, heat and oxygen have all been shown previously to cause degradation of these pigments (Oliver & Palou 2000). This study identified additional parameters that must be considered when attempting absolute quantification of chlorophylls and carotenoids, and includes: the inherent inaccuracy in normalisation to an externally added internal standard; the loss of pigments caused by freeze-drying tissue prior to extraction; and the detrimental effect of concentrating the extraction prior to HPLC analysis. Pheophytin *a* was identified as an indicator of degradation, and its formation was systematically minimised through specific optimisation steps. The optimised single step extraction protocol is able to extract photosynthetic pigments from grape berries and leaves that differed in age and developmental stage. The following pigments could be absolutely quantified after HPLC separation: chlorophyll *a*, chlorophyll *b*, lutein, β-carotene, zeaxanthin, antheraxanthin, violaxanthin and neoxanthin.

As research strategies shift from a candidate gene approach towards an "omics" approach the limitations in metabolomics have become apparent. While researchers are accustomed to large quantities of accurate data being generated from genomic and transcriptomic studies, the same is not true of metabolomic data (Sumner et al. 2003). However, the metabolome is the final functional level of all the "omic" levels and can be considered the biochemical phenotype of the organism (Ryan & Robards 2006). If accurately quantified, metabolite levels provide a more comprehensive insight into the biochemical, cellular and physiological processes occurring in cells, organs and ultimately plants. Due to the diversity and vast amount of metabolites present in a plant, there is currently no single analysis capable of quantifying (or even profiling) the entire metabolome of a plant. The partial analysis of a metabolome is, however, practical when performing studies directed to towards the manipulation of specific metabolic pathways. The method described in Chapter 3 provides

efficient, reproducible and accurate quantification of chlorophylls and carotenoids contributing to our understanding of the grapevine metabolome.

Transcriptional analysis of the transgenic population showed a 12-fold difference in *VvCCD1* expression in leaves. Despite successful manipulation of *VvCCD1* transcript levels, metabolite analysis showed no correlation between carotenoid or apocarotenoid concentrations and gene expression. Assuming a positive correlation between *VvCCD1* transcripts and protein levels it appears that one or more forms of post-translational regulatory mechanisms may be involved.

Additionally, *VvCCD1* transcript levels in leaves appear to be controlled by post-transcriptional gene silencing (PTGS). The majority of the transgenic lines transformed with an overexpression cassette did not show a significant increase in total *VvCCD1* expression when compared to wild-type lines. Although, in lines where no increase in total *VvCCD1* expression was observed, subsequent analysis confirmed that transcripts from both endogenous and transgenic *VvCCD1* contributed to the total expression. The plants, therefore, seem to have compensated for the increased *VvCCD1* expression by reducing the total transcript levels to levels similar to those found in wild-type lines, presumably through a form of PTGS. While transcriptional silencing of genes in overexpression studies has previously been observed, further research is required to identify the specific mechanism responsible for this phenomenon. RNA polymerases that have been shown to act specifically on transgene-derived RNA are described by Dalmay et al. (2000).

Control over enzyme action may be maintained through sub-cellular compartmentalisation, whereby access of VvCCD1 to the carotenoid substrates is limited. Carotenoid substrates are predominantly found in the plastids while VvCCD1 is localised to the cytosol. This division would possibly be relaxed in reproductive organs, such as grape berries. Apocarotenoid levels have been observed to increase in ripening berries one week after véraison, despite *VvCCD1* expression peaking at véraison (Mathieu et al. 2005). After véraison the berry stops actively photosynthesising and chloroplast degradation occurs. This, coupled with the high levels of osmotic stress in ripening berries, would likely result in leaky membranes and exposure of VvCCD1 to the liberated carotenoid substrates.

Although metabolite analysis of the transgenic population showed no correlation between apocarotenoid concentrations and gene expression, correlations were found within specific apocarotenoid pools. Most noteworthy geranylacetone correlated with MHO, and similarly β-ionone with α-ionone, but no correlation between the two pools. This suggests that a division between the availability of the respective carotenoid substrates exists. This division could be based on either enzyme access or enzyme-substrate specificity. It is possible that plastid-localised enzymes differentially cleave these carotenoids resulting in apocarotenoids that are subsequently transported to the cytosol. These apocarotenoids are then subject to further cleavage by VvCCD1. Floß et al. (2008) showed that silencing of *Medicago truncatula CCD1* in roots led to an increase in C_{27} apocarotenoids, but no significant change in the concentration of carotenoids. They concluded that the C_{27} apocarotenoids are the primary substrate for MtCCD1, and suggest that an AtCCD7 orthologue is able to asymmetrically cleave carotenoids in the plastid forming C_{27} apocarotenoids that may be transported to the cytosol.

It is important to note that data for the characterisation of the *in planta* function of *VvCCD1* was obtained from analysis of leaf tissue. Leaves are the primary site of photosynthesis in plants and therefore it is not surprising that the carotenoid pathway is tightly regulated, as manipulation of these crucial pigments could lead to a non-viable plant. Future studies on grape berries from the transgenic lines generated in this study will be of great importance in further elucidation of the *in planta* function of *VvCCD1*. Berries, the reproductive organs of grapevine, accumulate apocarotenoid-derived flavour and aroma compounds (Baumes et al. 2002), and have little need for tight regulation of photosynthetic pigments as ripening proceeds.

In conclusion, the aims of this study were met and the findings contribute to the current knowledge of CCD1 functioning *in vitro* and *in planta* (specifically in photosynthetic tissue). The transgenic lines generated serve as a genetic resource for the further study of carotenoid and apocarotenoid metabolism. This work has shown that the pathway is under tight regulation at multiple molecular levels: transcriptional, post-transcriptional, and post-translational. Further elucidation of the control mechanisms regulating this pathway, therefore, requires a systems biology approach. Successful metabolic engineering of this economically important pathway requires not only knowledge of the carotenoid biosynthetic enzymes, but a fundamental understanding of the regulatory mechanisms at work.

5.2 References

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